CHEMICAL, BIOLOGICAL AND SENSORY CHARACTERIZATION OF HOPS AND DRY-HOPPED BEERS: PERSPECTIVES FOR THE USE OF PORTUGUESE GENOTYPES

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À minha mãe, pai, madrinha, irmã, esposa e filho

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ABSTRACT

The craft breweries motived fundamental changes in the beer market. Nowadays, not only microbreweries, but also big companies, are interested in the production of more flavoured beers, with intense aroma and bitterness, and hops have been a key ingredient in this trend. Brewers extract different flavours from the plant by adding higher hop quantities, by using different varieties, and by adding hop at different phases of beer production. Consequently, year after year, hop companies have intensified investments expanding planted area and breeding, to release new varieties that supply the market with differentiated products, promoting high diversity in hop flavour. For that purpose, native plants are often used for breeding. Centre and north regions of Portugal have an extensive population of native hops that present a large morphologic variability. Those can be an alternative for the industry, being necessary their characterization at genetic, sensorial, and chemical levels. The goal of this thesis was to provide new insights about chemical, biological and sensorial characterization of hops and dry-hopped beers and search for new perspectives concerning the use of native Portuguese hops. A total of 178 hop samples were analysed, 58 commercial varieties and 120 Portuguese natives, including 97 samples provided by Banco Português de Germoplasma Vegetal and 23 samples collected in nature.

Hops discrimination and characterization was carried out, comparing Portuguese native hops and commercial varieties by DNA, infrared, gas chromatographic and sensory analyses. For the genetic discrimination, hops were genotyped by high resolution melting analysis (HRMA) of a minimal 7 markers set of single nucleotide polymorphic (SNP). Results showed a great diversity amongst accessions and a clear cluster separation from most of the commercial references used, demonstrating the potential genetic richness of the Portuguese germplasm. Vibrational spectroscopic techniques near infrared (NIR) and mid-infrared (MIR) spectroscopy demonstrated to be cost-effective, non-destructive, environmentally friendly, and fast alternative methods to discriminate and verify authenticity of hops. Volatile profiles were explored using headspace-solid phase micro-extraction and gas-chromatography mass-spectrometry (HS-SPME-GC-MS). Most of Portuguese hops were grouped, separately from commercial varieties, 12 volatile compounds were identified as main responsible for the separation. At the sensorial level, samples were evaluated by a semi-trained sensory panel. Portuguese hops presented wide sensory characteristics. In general, it was more resinous, spicy and herbal than control commercial hops used (European and North-American varieties), which were more citrus, fruity/ sweet, and floral. Despite a few exceptions, Portuguese native hops globally presented statistical differences from commercial references for all the parameters analysed.

Sensory impression and odour-active volatile composition of hops and dry-hopped beers from four selected Portuguese wild hop genotypes was studied. The quantification of key odorant compounds on dry-hopped beers provided relevant information to elucidate about the brewing characteristics of the selected hops. Specific descriptors were in agreement with threshold analysis of those key odorant compounds. Addittionaly, Beers dry-hopped with the “Special Flavour” fruity-citrus Mandarina Bavaria variety were evaluated along the time to understand the transference of volatile compounds and the relationship with sensorial characteristics. It was possible to find successful models to predict sensory characteristics of dry-hopped beers by equation regressions considering the content of only four volatile compounds (myrcene, linalool, 2-methylbuthyl-2-methylpropanoate, and α-humulene).

A wide range of pharmacological properties have been described for hop and hops derivatives, namely, antioxidant, anti-inflammatory and anticancer-related activities. Therefore, the bioactivity of beers can be influenced by hops usage, which in other hand can be relevant for the production of functional products. Therefore, the extraction of bioactive compounds was studied, and a model to optimize and predict the extraction of α-acids and xanthohumol and reach the highest yield of extraction in dry-hopped beers was proposed. Regression models were established to determine the maximum efficiency extractions, which were reached at 2 weeks with dose rates of 147 mg/ L of α-acids and 13.9 mg/ L of xanthohumol. Both compounds were also included in a study concerning the antiproliferative activity of the main bioactive compounds of hops. Xanthohumol, isoxanthohumol, iso-α-acids and a mixture of α- and β-acids were tested concerning its proliferation inhibition against colon adenocarcinoma Caco-2 cells. Xanthohumol did not show antiproliferative activity at maximum concentrations of 20 μg/ mL, however isoxanthohumol, the mix of α/β-acids, and iso-α-acids, showed antiproliferative activities, both, as pure compounds, and as part of the beer matrix with a synergist.

Overall, a large population of Portuguese native hops was characterized, and presented different characteristics. Being promising for the development of new varieties and new beer products, favouring the diversity not only of Portuguese, but also the worldwide brewing sector. In addition, new knowledge was achieved concerning the extraction of main compounds from hops in dry-hopping process, which are of high importance on organoleptic and bioactive characteristics.

Keywords: hops, Portuguese hops, wild hops, beer, dry-hopping, hop compounds.

RESUMO

Atualmente, o setor de produção de cerveja (incluindo os maiores produtores) investe na obtenção de cervejas diferenciadas, principalmente ao nível do sabor e aromas. Neste contexto, o lúpulo tem sido um ingrediente-chave dessa tendência. Os cervejeiros têm conseguido produtos inovadores aumentando a quantidade de lúpulo, usando variedades inovadoras, e adicionando lúpulo em diferentes fases da produção de cerveja. Consequentemente, ano após ano, as empresas produtoras de lúpulo têm intensificado investimentos em prol da expansão de áreas plantadas, afim de suprir a necessidade do mercado, incluindo o desenvolvolvimento de novas variedades, com diferentes propriedades químicas, promovendo a diversidade no setor. Neste contexto, plantas nativas, silvestres, são frequentemente valorizadas para desenvolvimento de novas variedades. As regiões centro e norte de Portugal possuem uma extensa população de lúpulos nativos, que apresentam uma grande variabilidade morfológica. Estes podem ser uma alternativa para a indústria, tornando-se assim necessária a sua caracterização genética, sensorial e química. Desta forma, o objetivo do presente trabalho foi caracterizar química, biológica e sensorialmente o lúpulo nativo de Portugal, assim como as cervejas produzidas por técnicas de *dry-hopping*. Para tanto, foram analisadas 178 amostras de lúpulo, 58 variedades comerciais e 120 lúpulos nativos, incluindo 97 amostras fornecidas pelo Banco Português de Germoplasma Vegetal e 23 amostras recolhidas na natureza.

Foi efetuada a discriminação e caracterização, comparando os lúpulos nativos e as variedades comerciais através da análises de ADN, do espectro de infravermelho, por cromatografia gasosa e análise sensorial. Para a discriminação genética, os lúpulos foram genotipados por análise de fusão de alta resolução de fragmentos (HRMA) com um mínimo de 7 marcadores de polimorfismo de nucleotídeo único (SNP). Os resultados mostraram uma grande diversidade entre as amostras, e uma clara separação da maioria das referências comerciais utilizadas, demonstrando a potencial riqueza genética do germoplasma Português. Técnicas espectroscópicas vibracionais no infravermelho próximo (NIR) e infravermelho médio (MIR) revelaram ser métodos alternativos, eficazes em termos de custo, não destrutivos, sustentáveis, e rápidos para discriminar e verificar a autenticidade dos lúpulos. Perfis de voláteis foram explorados usando micro extração em fase sólida e cromatografia gasosa, acoplado a espectrometria de massa (HS-SPME-GC-MS). A maioria dos lúpulos Portugueses agruparam-se distintamente das variedades comerciais, tendo sido identificados 12 compostos como os principais responsáveis pela separação. As amostras foram também avaliadas sensorialmente por um painel semi-treinado. Os lúpulos Portugueses apresentaram amplas características sensoriais, no geral, predominaram aromas resinosos, condimentados e herbáceos, enquanto os lúpulos

comerciais avaliados (variedades europeias e norte-americanas) apresentaram-se mais cítricos, frutados/adocicados e florais. De forma geral, os lúpulos nativos de Portugal apresentaram diferenças estatísticas das referências comerciais, para todos os parâmetros analisados.

Seguidamente foi aprofundado o conhecimento a nível sensorial, e dos respectivos compostos voláteis, de uma seleção de quatro lúpulos nativos Portugueses e correspondentes cervejas após *dry-hopping*. A quantificação dos principais compostos voláteis nas cervejas, , forneceu informações relevantes para elucidar as propriedades dos quatro lúpulos selecionados quando utilizados em *dry-hopping*. Houve concordância entre descritores sensoriais e a análise quantitativa dos compostos, levando-se em consideração os limiares de perceção (*thresholds*) dos principais compostos odoríferos. Cervejas produzidas com variedade comercial Mandarina Bavaria, conhecida por ser frutada e cítrica, foram avaliadas ao longo do tempo para um melhor conhecimento da transferência de compostos voláteis, e sua correlação com as características sensoriais. Foi possível encontrar modelos para previsão de características sensoriais por meio de equações de regressão, considerando apenas a quantificação de quatro compostos voláteis (mirceno, linalol, 2-metilbutil-2-metilpropanoato, e α-humuleno).

Uma ampla gama de propriedades farmacológicas foi descrita para os lúpulos e seus derivados. Nomeadamente, as propriedades antioxidantes, antiinflamatórias e anticancerígenas. Consequentemente, a bioatividade das cervejas é influenciada pelo uso do lúpulo, o que pode ser relevante para a indústria cervejeira, a fim de produzir produtos funcionais. Neste contexto, a extração de compostos bioativos foi estudada, tendo sido propostos modelos para otimizar, prever, e maximizar o rendimento de extração de α-ácidos e xanthohumol. Uma tendência geral foi observada indicando uma correlação positiva entre a quantidade de lúpulo adicionada e os níveis de ambos os compostos em cervejas. As máximas eficiências de extrações foram atingidas em 2 semanas com taxas de dose de 147 mg/ L de α-ácidos e 13,9 mg/ L de xanthohumol. Ambos os compostos foram incluídos no estudo sobre a atividade antiproliferativa dos principais compostos bioativos do lúpulo. Xanthohumol, isoxantohumol, iso-α-ácidos e uma mistura de α- e β-ácidos foram testados quanto à capacidade de inibição da proliferação contra células Caco-2 de adenocarcinoma do cólon. Nas concentrações testadas, o xanthohumol (até 20 μg/ mL) não apresentou atividade antiproliferativa, entretanto o isoxantohumol, a mistura α / β-ácidos, e iso-α-ácidos, apresentaram atividades antiproliferativas, como compostos puros ou integrados na matriz cerveja onde se notou um possívelefeito sinérgico.

No geral, um grande número de populações de lúpulo nativo português foi caracterizado, apresentando uma considerável diversidade a vários níveis. Os lúpulos caracterizados podem considerar-se promissores para o desenvolvimento de novas variedades e novos

produtos de cerveja, favorecendo a diversidade do setor não só a nível do país , mas também mundial. Além disso, novos conhecimentos foram alcançados em relação à extração dos principais compostos do lúpulo no processo de *dry-hopping*, importante em propriedades organoléticas e na bioatividade da cerveja.

Palavras-chave: lúpulo, lúpulos Portugueses, lúpulos nativos, *dry-hopping*, compostos do lúpulo

LIST OF PUBLICATION

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Machado Jr, J. C.; Faria, M. A.; Ferreira, I. M. P. L. V. O.; Páscoa, R. N. M. J.; Lopes, J. A. Varietal discrimination of hop pellets by near and mid infrared spectroscopy. Talanta. 2018; 180: 69 - 75.

Machado Jr, J. C.; Faria, M. A.; Melo, A.; Ferreira, I. M. P. L. V. O. Antiproliferative effect of beer and hop compounds against human colorectal adenocarcinome Caco-2 cells. Journal of Functional Foods. 2017; 36: 255 - 261.

Book chapter

Machado Jr, J. C.; Faria, M. A.; Ferreira, I. M. P. L. V. O. Hops new perspectives for an old beer ingredient. In: Grumezescu, A., M.; Holban, A. M. Emerging trends and developments in beverage science. 1st Ed. Bucharest: Elsevier; 2019. v 10.

Submitted articles

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Oral comunications

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Júlio C. Machado Jr., Zita E. Martins, and Isabel M.P.L.V.O. Ferreira. Desenvolvimento de novas cervejas: análises químicas e sensoriais focadas nos compostos e atributos do lúpulo. In II Jornadas do Lúpulo e da Cerveja. Bragança, Portugal, 18-19 July 2019.

Júlio C. Machado Jr., Florian Lehnhardt, Miguel Faria, Hubert Kollmannsberger, Martina Gastl, Thomas Becker, and Isabel M.P.L.V.O. Ferreira. Characterizing the diversity of wild hops (Humulus lupulus) from Portugal. In 37th Congress EBC European Brewery Convention, Antwerp, Belgium, 2-6 June 2019.

Júlio C. Machado Jr. Aroma dos alimentos: cerveja. In Pint of Science Portugal. Bragança, Portugal, 20-22 May 2019.

Júlio C. Machado Jr., Zita E. Martins, Sara C. Cunha, Isabel M.P.L.V.O. Ferreira. Correlation of aroma characteristics with volatile profile of hops. In XXIV Encontro Luso-Galego de Química, Porto, Portugal, 21-23 November 2018.

Poster comunications in conferences

Julio Cesar Machado Junior, Miguel A. Faria, Ana Maria Barata, Andreja Cerenak, Isabel M.P.L.V.O. Ferreira. Portuguese wild hops genotyping by HRMA of a minimal SNP set. In 37th Congress EBC European Brewery Convention, Antwerp, Belgium, 2-6 June 2019.

M.A. Faria, Jr. J.C. Machado, A. Melo, I.M.P.L.V.O. Ferreira. Antiproliferative activity of hop-derived compounds against human colon cancer Caco-2 cells. In 36th Congress EBC European Brewery Convention, Ljubljana, Slovenia, 14-18 May 2017.

Jr. J.C. Machado, M.A. Faria, A. Melo, I.M.P.L.V.O. Ferreira. Evaluation of a-acids content of beer produced by dry hopping techniques. In 36th Congress EBC European Brewery Convention, Ljubljana, Slovenia, 14-18 May, 2017.

A. Melo, Jr. J.C. Machado, M.A. Faria, C. Mansilha, I.M.P.L.V.O. Ferreira. Impact of dry hopping techniques on xanthohumol content of beer. In 36th Congress EBC European Brewery Convention, Ljubljana, Slovenia, 14-18 May 2017.

A. Melo, Jr. J.C. Machado, A.A. Capitão, M. Cancela, A.I. Ribeiro, C. Mansilha, I.M.P.L.V.O. Ferreira, Xanthohumol evaluation in hop gun dry hopping techniques, in 2nd International Conference on Food Chemistry and Technology, Las Vegas, NV, US, 14 to 16 of November 2016.

Machado Junior, Julio Cesar; Cunha, S. C.; Morais, J. S.; Isabel M.P.L.V.O. Ferreira. Volatile profile of wild hops grown in the North of Portugal: Comparison with a cultivar hop produced in the same region. In: FOODINTEGRITY 2016, Praga. Assuring the integrity of the food chain: Fighting Food Fraud. Prague: University of Chemistry and Technology Prague, 2016. v. April. p. 136 - 136

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LIST OF ABBREVIATIONS

2MB2MP - 2-methylbutyl 2-methylpropanoate

ABA - α and β-acids

AFLP - Amplified Fragment Length Polymorphism

AHC - Agglomerative Hierarchical Clustering

ANOVA - Analysis of variance

ASBC - American Society of Brewing Chemists

ATR - Attenuated Total Reflectance

AUROC - area under the ROC

BPGV - Banco Português de Germoplasma Vegetal

C - Cones

CAMRA - Campaign for Real Ale

CATA - Check-All-That-Apply

Ch - Chapter

CM - Culture media

CPCC - Cophenetic correlation coefficient

DAD - Diode array detection

DArT - diversity array technology markers

DMEM - Dulbecco's modified Eagle's medium

DMSO - dimethyl sulfoxide

DMTS - dimethyl trisulphide

DVB/ CAR/ PDMS - Divinylbenzene/ carboxen/ polydimethylsiloxane

EBC - European Brewery Convention

FBS - Fetal bovine serum

FTIR - Fourier-Transform Infrared

GC - Gas chromatography

GC-O - Gas chromatography-olfactometry

HCA - Hierarchical cluster analysis

HPLC - High-performance liquid chromatography

HRMA - High resolution melting analysis

HS - Headspace

IAA - iso-α-acids

IBU - International bittering unit

ICE - International Calibration Extract

IHGC - International Hop Growers' Convention

IHPS - Inštitut za hmeljarstvo in pivovarstvo Slovenije

InGaAs - indium-gallium-arsenide

IPA - India Pale Ale

IS - Internal standard

ITEX - In-tube extraction

IXN - Isoxanthohumol

L - Leaves

LV - Latent variables

MIR - Vibrational spectroscopic techniques mid-infrared

MS - Mass spectrometry

MTT - 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

NASS - National Agricultural Statistics Service

NDH - no dry-hopped

NGS - Next Generation Sequencing

NIR - Vibrational spectroscopic techniques near infrared

NIST - National Institute of Standards and Technology

P - Pellets

PCA - Principal Component Analysis

PCR - Polymerase chain reaction

PLS - Partial least squares

PLS-DA - Partial least squares discriminant analysis

PLS-R - Partial Least Squares regressions

QDA - Quantitative Descriptive Analyses

RAPD - Random Amplified Polymorphic

RI - Retention index

RMSE - Root-mean-square error

ROC - Receiver Operator Characteristic

RP- HPLC - reverse-phase liquid chromatography

RT - Retention time

SD - Standard deviation

SNP - Single nucleotide polymorphic

SNV - Standard normal variate

SPE - solid phase extraction

SPME - Solid phase micro-extraction

SSR – Simple Sequence Repeats

Th - Thresholds

TPC - Total polyphenols content

USDA - United States Department of Agriculture

UV - Ultra-violet

VIP - Variable importance in projection

XN - Xanthohumol

GENERAL SCOPE AND OBJECTIVES

Hop (*Humulus lupulus* L.) is one of the four main beer ingredients. It imparts bitterness, microbial protection, foam stability, together with specific flavours and aromas to beer (1), mainly due to the humulones (α-acids), lupulones (β-acids), and essential oils that are concentrated in the lupulin material from the inflorescences of female plants (2). The selection of hop's variety has become a main target for brewers that want to produce different and exquisite beer styles.

New tendencies are observed nowadays on hop uses, due to the increased consumption of craft beers (that contain more hop per litre of beer), and the use of dry-hopping techniques. This practice of adding hop at cold stages of beer production, imparts more hoppy flavours to those beers (1). Motived by this new trends, the world production area and market price of established commercial hops (3) has increased. Not only, the old typical hops are largely produced, but a wide range of new hop varieties are used nowadays, in most cases those varieties resulted from the breeding of commercial and native (wild) plants (4, 5). More than 260 varieties are presently cultivated worldwide (6, 7). These differ qualitatively and quantitatively in their chemical composition, including the essential oils (characterized by terpenes, alcohols, acids, esters, ketones and aldehydes) and, in their non-volatile fraction (composed by polyphenols, α- and β- acids) (8).

Several high valued hops had been launched in the market at differentiated prices, making its authentication crucial for brewers. In this context, fraudulent declarations or labelling of hop products is an issue that should not be excluded. Total or partial replacement of expensive hops with less expensive ones results in economic benefit to dealers as it was reported recently (9). Currently the most consensual technology for varietal discrimination of hops is based on the use of 25 microsatellite DNA markers that can assure accurate genotyping, i.e. the complete discrimination, of the known varieties (10). However, this methodology requires advanced and expensive equipment, which motivates the search for more expedite techniques, such as the use of a single nucleotide polymorphism (SNP) analysis using real time polymerase chain reaction (PCR) or vibrational spectroscopic techniques, namely, near infrared (NIR) and mid-infrared (MIR) spectroscopy. Those emerge as suitable alternatives for hops differentiation and identification targeting authenticity purposes. Notwithstanding, they require previous validation before bring used as a standardized tool to authenticate commercial samples and/or evaluate new hop varieties.

The breeding of new varieties is often obtained from unexplored native plants (4, 5) and Portugal has a wide population of wild plants that can be explored as a new possibility for hops market. More than a hundred of native hop populations have already been identified

and representative genotypes are presently maintained as field collection in the Banco Português de Germoplasma. Their morphologic characterization revealed large variability among them (11). Additionally, this collection can be increased as more hop samples are easily found near bank rivers, mainly in the north and centre of Portugal. Despite that, no published studies were found about odour-active compounds and sensory characteristics of those Portuguese native hops.

Key odour-active hop compounds, include methyl butyl esters (fruity), ketones (fruity, citrus and floral), terpenes (herbal, woody, spicy and terpenic), methyl thioesters (sulphur and fruity), monoterpenoid alcohols (floral and citrus), monoterpenoid esters (fruity, greenery and floral), and cinnamate esters (fruity and balsamic) (1, 2, 12). Several studies have been done to investigate hops volatile profile and their impact on beer sensory characteristics (1, 13, 14). However, the hop composition varies significantly, which requires more studies concerning the extraction of volatile compounds from hop to beer. Dry-hopping is widely applied to impart into beer an aroma that is close to the original hop aroma. Besides the selection of hop variety or the use of hop mixtures, other variables must be controlled on dry-hopping practices, namely, hop amount, time of contact, raw material form (whole hops, fresh and dried cones, pellets or extracts), temperature, and moment of addition (during or after first or second fermentations) (15-19).

In addition to flavour, bitterness, and foam stability, hops and their derivatives present a wide range of pharmacological properties, particularly, microbial protection, antioxidant, anti-inflammatory, anticancer-related, and sedative properties due to α-acids, β-acids and prenylflavonoids (xanthohumol, isoxanthohumol and 6- and 8-prenylnaringenin). Those compounds that occur almost exclusively in hops (20) have been linked with beneficial health effects and regulation of some diseases (21, 22). However, during the boiling phase isomerization of xanthohumol to isoxanthohumol and α-acids to iso-α-acids (23, 24) occurs. Nevertheless, dry hopping techniques can be used for enrichment of beers with those beneficial phytochemicals present in hop, even though, more studies are needed to understand the effects of enriched beers on health.

The work described in this thesis was conducted to provide new insights about chemical, biological and sensory characterization of hops and dry-hopped beers and search for new prospectives concerning the use of native Portuguese hops and their behaviour in beer production. Emphasis was given to dry-hopping techniques to optimize and predict the extraction of volatiles and bioactive compounds (α-acids and xanthohumol). To reach these objectives, specific goals were defined as quoted below:

- Use 7SNPs to genotype the population of Portuguese native hops, comparing with reference commercial varieties.

- Evaluate the suitability of near- and mid-infrared vibrational spectroscopy to discriminate different hop varieties and assess the potential of these methods to be used as an authenticity tool of hop varieties.

- Evaluate the volatile profile and aroma attributes of Portuguese native hops and search for new genotypes with appropriate characteristics for the modern beer trends.

- Assess the sensory impression and odour-active volatile composition of hops and dry-hopped beers from selected Portuguese wild hop genotypes.

- Predict the extraction of volatile compounds, from a “special flavour” hop variety and the impact on sensory characteristics and volatile compounds composition of dry-hopped beers.

- Optimize and predict the extraction of α-acids and xanthohumol to reach the highest yield of extraction in dry-hopped beers, to answer consumer’s demand for beer with higher bioactivity.

- Evaluate, *in vitro,* the antiproliferative activity of xanthohumol, isoxanthohumol, α and β-acids, and iso-α-acids, both as pure compounds and as part of the beer matrix on colon Caco-2 cells.

A structured and simplified representation of the main objectives and applied studies to be exploited in this thesis is presented in Figure I.

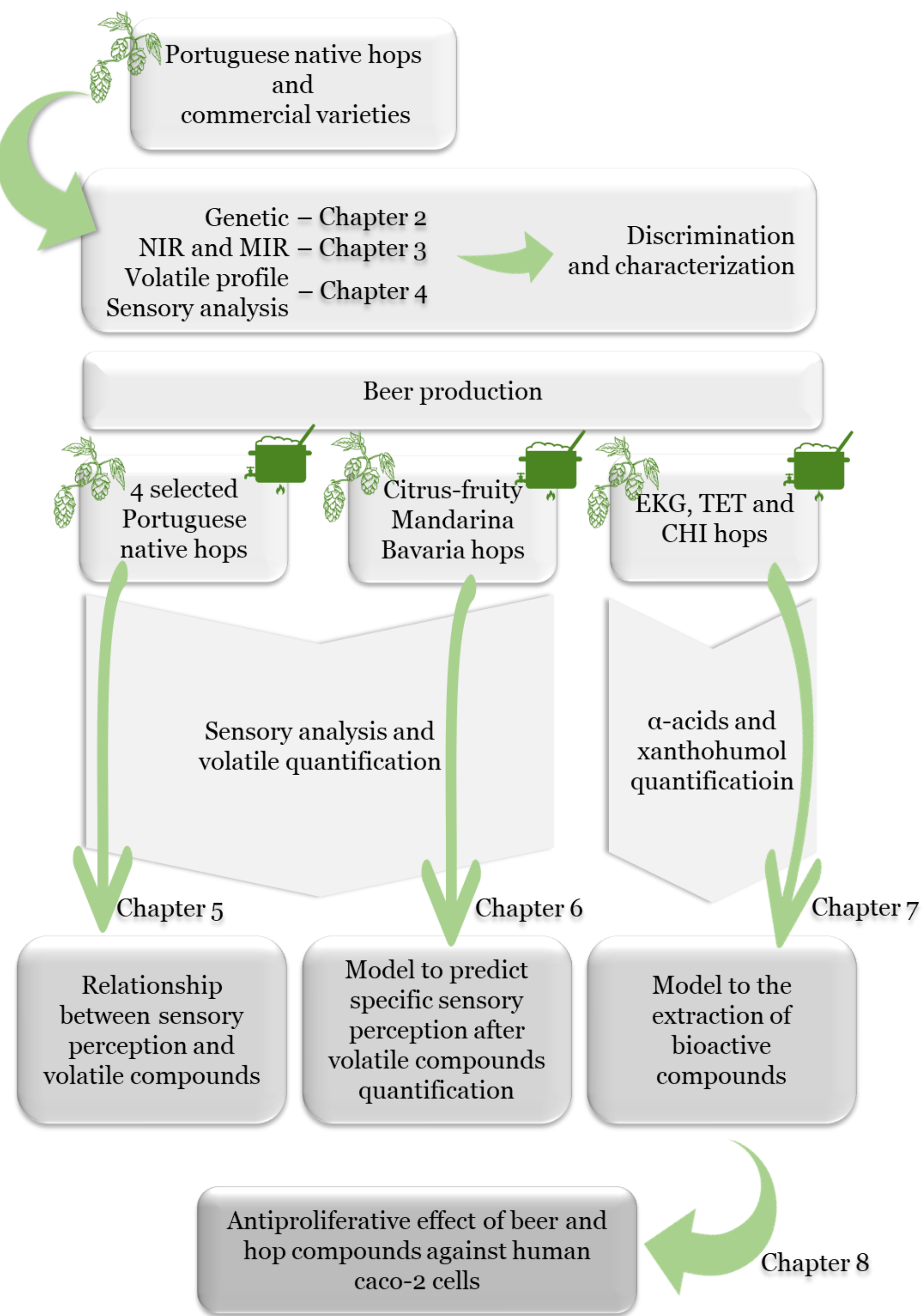


Figure I. Diagram of the main objectives and studies applied in the present work.

THESIS OUTLINE

This thesis is divided in seven parts and includes nine chapters (Figure I). **Part I** is composed by chapter 1 that presents a literature review concerning hop and its use in dry-hopped beers. This chapter answers questions, such as, what are hops? Which are hop bitter compounds, phenolics and volatiles? Hop varieties and new trends of hop uses? Current market? Beer production? Analytical methods used for hop and beer analysis.

Part II is related with hops discrimination/ characterization and includes chapters 2 to 4. Chapter 2 deals with genetic discrimination/ characterization of Portuguese native hops in comparison with reference hops. Chapter 3 describes discrimination of commercial and Portuguese native hops by near or mid infrared spectroscopy. Chapter 4 presents the discrimination of Portuguese native hops and commercial varieties by analyzing their volatile profile and relationship with odour characteristics.

Part III includes chapters 5 and 6, which are related with beer production and impact of hop in sensory characteristics and volatile compounds composition of dry-hopped beers. Chapter 5 is focused on beers produced using selected native Portuguese hops, whereas chapter 6 is focused on dry-hopped beers produced using a "special flavour" hop variety.

Part IV includes chapters 7 and 8 that are related with beer composition on bioactive compounds, such as, α-acids, xanthohumol and others and the impact of hop in beer composition and bioactivity. Chapter 7 is focused in modelling α-acids and xanthohumol extraction in dry-hopped beers, whereas chapter 8 describes the antiproliferative effect of beer and hop compounds against human colorectal adenocarcinoma caco-2 cells.

Part V presents the overall conclusions from this thesis as well as the future prospects. **Part VI** supplementary material. **Part VII** contains all the references cited throughout the thesis.

PART I

Literature review

CHAPTER 1

Hops Report: composition, market, new trends in beer production and research

1.1. Introduction

Hops are an essential beer ingredient that imparts bitterness and aroma. Moreover, they also contribute to the stability of beer foam and to beer preservation. More recently, hops usage is experiencing a new change driven by the internationally growing preference for more intensely flavoured beers, sustained by the craft brew sector. This movement has led to the introduction of much higher amount of hops in different phases of beer production and to the increasing search for new flavours. Some hop varieties received special attention, including few old typical hops, largely used until the present date, and intensive search for new varieties dubbed “Green Gold”, which also occurred. A large number of new exquisite varieties have been described and increasingly valued in the market. The global area of hop cultivation has increased in the last five years although the total world beer production decreased in the same period, which corroborates the trend of using more hops per litre of beer. Additionally, a wide range of pharmacological properties has been described for hop and hop derivatives, namely, antioxidant, anti-inflammatory and anticancer-related properties, which are of major relevance to the pharmaceutical industry. Beer bioactivity can be influenced by hops usage, which can configure an important tool for brewers aiming the development of functional products.

The aim of the present chapter is therefore to summarize the importance of hops in this new trend of beer production, reviewing hops market, varieties, forms and methods of utilization, composition, importance in the beer bioactivity and new discoveries in hops research.

1.2. What are hops?

Hops (*Humulus lupulus* L.) are perennial climbing plants of the Cannabaceae family Annually it grows during spring and summer but has an important wintering period of six to eight weeks of dormancy with temperatures below 4.4º C. It is a photoperiodic plant, needing around 15 hours of daylight, and preference of six to eight hours of sunshine, in both vegetative growth and flowering stages. The access to water is also very important, but not necessarily from rain, since the water absorption is done through the roots that cannot stay soaked. Hops prefer the soils around the river margins in the cold zones between latitudes 30° and 55°. The best thriving geographical region is between 40º and 50º of the northern and southern hemispheres, in Europe, centre and north of United States of America (US), Central Asia, Southern Africa, Argentina and south of Oceania (25, 26).

Hops are indigenous from the Northern Hemisphere, growing in Europe, Asia and North America. European wild hops have a wide geographical distribution across Eurasia, from

Portugal to Altai region, however, lower genetic variation was found on those hops (27), contrarylly to the verified in North American wild hops with high genetic diversity (28). Besides the common hop, the *Humulus* genus includes two other species, *Humulus japonicus* Siebold & Zucc and *Humulus yunnanensis* Hu, but only *H. lupulus* is of industrial and medicinal importance (25).

Hop is a dioecious plant, separated in two genders, but only the female ones develop cones (Figure 1.1). Hop flowers or cones resemble pine cones and are composed of thin, green, papery, leaf-like bracts. Bracts, and bracteoles (small bracts) are leaf like structures that surround the entire cone, attaching to a central axis. Underneath the bracteoles are the lupulin glands that contain the total resins and essential oils. Besides lupulin, hops cones are composed mainly of cellulose, lignin, water, proteins, monosaccharides, pectins, amino acids, lipids and wax (29).

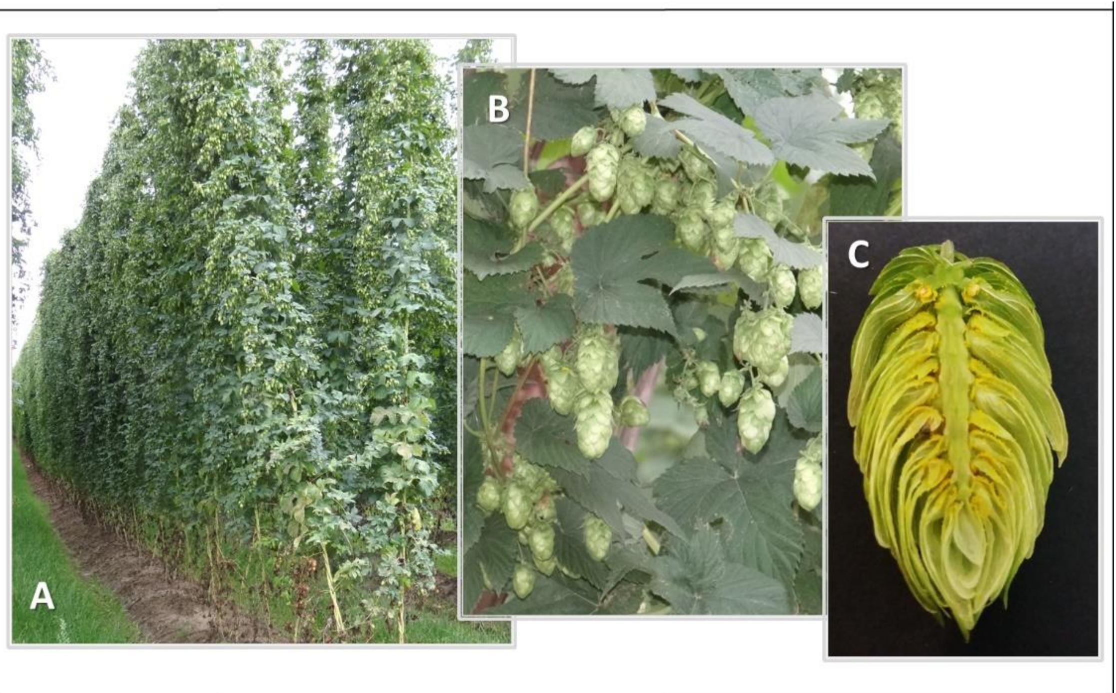


Figure 1.1. Different views of the hop plants: in intensive production field (A); cones ready to harvest (B); hop flower exposing lupulin glands (C).

Being hop cones one of the four main beer ingredients its chemical composition has been widely studied (30-32). Hydrophobic compounds from hop cones present bitterness, mainly due to the α- and β-bitter acids, whereas the characteristic hoppy aroma is provided by the essential oils (33), which are concentrated in lupulin. Consequently, hop is responsible for the bitterness and the specific flavours and aromas of different beer styles.

Hop cones also contain biologically active phenolic compounds, which contribute to the preservation and stabilization of the organoleptic characteristics of the beverage, mainly due to its antioxidant, anti-microbiological and foam stabilization properties.

Different fractions can be extracted from hop cones and categorized according to their physicochemical properties (Figure 1.2). The essential oils are, by definition, the fraction of hops that can be isolated by steam distillation. The monoterpene, myrcene and the sesquiterpenes, α-humulene and β-caryophyllene, make up the bulk of the essential oil, together with great number of other terpenes, as linalool, farnesene, limonene, pinene and geraniol. Other volatile compounds, such as, alcohols, acids, ketones and aldehydes contribute to beer flavour, being quality parameters, and trade preferences of different hop cultivars (34). Non-volatile hop resins are characterized by their solubility in cold methanol and diethyl ether. They can be divided according to their solubility in hexane. The insoluble portions are the hard resins, which contain prenylated chalcones and flavanones, such as xanthohumol and prenylnaringenin. However, oxidation products derived from α and/or β-acids were also identified as hard-resin components (35). Soft resins are soluble in hexane and contain mainly prenylated phloroglucinol derivatives, such as the α and β-acids (8).

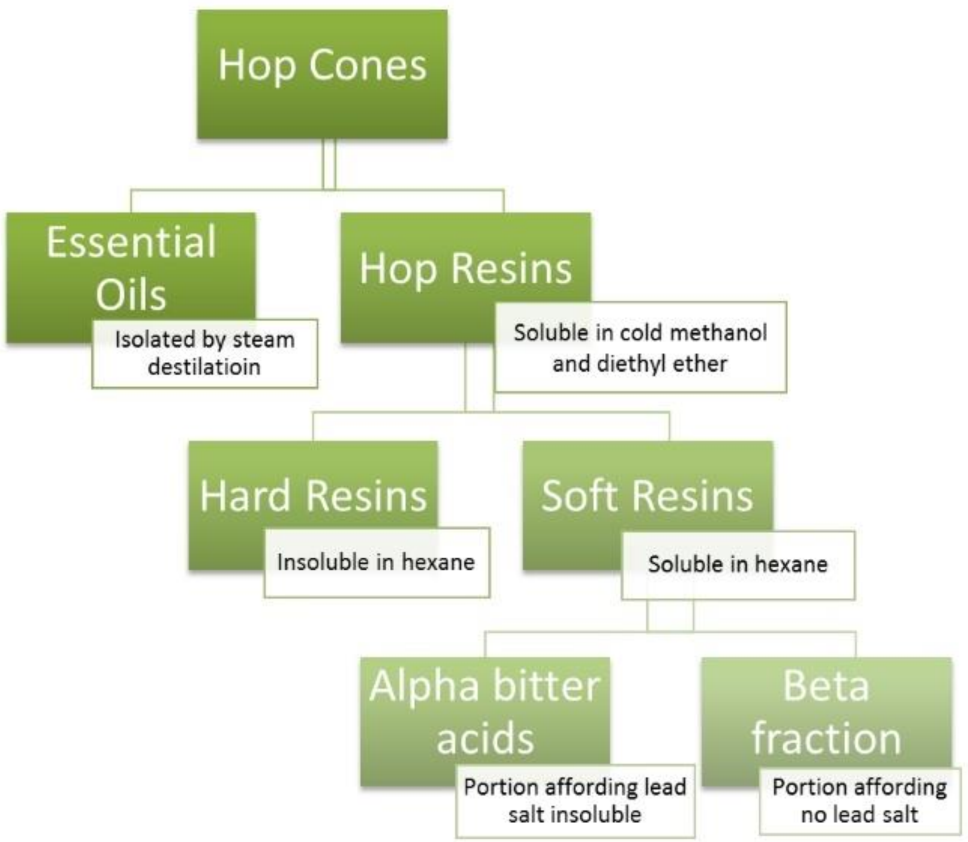


Figure 1.2. Scheme summarizing the separation of hop fractions extracted from hop cones.

Commercially hops are usually classified according to the concentration of α-acids. Bitter purpose hops contain more than 5% of α-acids, whereas aroma hops, appreciated mainly by their aromatic profile, present, on average, an amount of α-acids up to 5%. If the amount of α-acids exceed 10 % by weight the bitter varieties are classified as high alpha (36). Some bitter hops are also rich in flavour compounds, these varieties have been classified as dual purpose hops, because they have been used to bitter and also to add aroma to beers (37).

The majority hop crops are used in the brewing industry, as the ingredient that gives characteristic bitterness and aroma to beer. However, its health-promoting effects are well known and hop is used in folk medicine from ancient times. Likewise, the wide range of pharmacological properties described for hop, justifies that besides brewing, hops and their derivatives are also of interest to the pharmaceutical industry. Particular attention is given to prenylflavonoids (xanthohumol, isoxanthohumol and 8-prenylnaringenin) from hard resins that occur almost exclusively in hops and are considered as the most active phytoestrogens known (20). Hop oils and resins present sedative and other neuropharmacological properties. The main compounds responsible for sedative effects are bitter resins and essential oils. Interesting antibacterial and antifungal activities were also reported for β-bitter acids and lupulones, which present a strong antibacterial effect (38). Recent studies describe the effects of alpha bitter acids blocking the development of a number of complex lifestyle diseases that are collectively named as “metabolic syndrome” (39). Hops are therefore an interesting and rich source of compounds with applications either in the food industry or in the improvement of human health, due to its pharmacological properties. A brief description of the most relevant groups of compounds is presented below.

1.2.1. Soft resins: the bitter compounds

Soft resins are hops most studied compounds and assume a paramount importance concerning the bitterness intensity of hops and beer. They are constituted mainly by the prenylphloroglucinols, humulone and lupulone, together with their derivatives, also known as α and β-acids, respectively (40, 41). Bitter compounds from the soft resins are described as the main responsible for beer bitterness, although nowadays it is not clear whether additional hop components are required to the perception of the complex bitter profile of beer. This issue is presently one of the main active areas within beer research community.

Humulones, also known as α-acids, are part of the soft resins and consist of diprenylated phloroglucinol derivatives with variable acyl side. While *n*-humulone, *co*humulone and *ad*humulone are the major α-acids in all hop cultivars, others different derivatives have

been identified, such as *post*humulone, *pre*humulone and *adpre*humulone whose molecular structures are shown in Figure 1.3 (42).

Figure 1.3. Chemical structures of some soft resin components, α and β-bitter acids, and their isomerization (worth boiling) and oxidation (natural peroxidation) products.

The α-acids are direct precursors of the main bittering compounds from beer. During the wort boiling of brewing process, these thermally isomerize into iso-α-acids via an acyloin-typering contraction, resulting in the generation of two epimeric isomers: cis-iso-α-acids and trans-iso-α-acids. Iso-α-acids are mainly responsible for the typical bitter taste of beer, as well as the stability of beer foam and beer preservation properties, due to the antibacterial properties presented by isohumulones (2, 8, 43-45).

The residue obtained after isolating the α-acids from a hop extract contains the β-acids fraction that presents a structure highly similar to the α-acids, consisting in the triprenylated analogues of the α-acids (Figure 1.3). The term lupulone is used to identify individual β-acids; a similar nomenclature to that used for the α-acids is used to account for their varying acyl side chains, such as *co*lupulone, *n*-lupulone, and *ad*lupulone (8). The β-acids are considered virtually irrelevant for the brewing industry. Having an extra isoprenyl side chain they are significantly more hydrophobic than the α-acids, and practically

insoluble in aqueous media, which is the beer matrix (42). However, the β extract can be used as a raw material for the production of industrial bittering components, either by the transformation of the β-acids into the synthetic iso-α-acids or by their oxidation into the hulupones.

Humulinones and hulupones are other two important groups of compounds that are formed by the oxidation of α and β-acids, respectively. Although they were discovered a long time ago (46, 47) the interest in these compounds returned. Recent findings have demonstrated that humulinones and hulupones have a great potential for bitterness, even less bitter than iso-α-acids, they are more polar, therefore more soluble in beer, therefore presenting significant impact on beer bitterness (48, 49).

The concentration of α-acids varies from low values, as 2 to 5% of total dry weight to up than 10% reaching values as high as 14 to 20% in the high alpha varieties. β-acids generally appear in lower concentration than α-acids, on average ranging about 4 to 6% of dry weight, however may vary up to 10%. Humulinones are present in hops most commonly at a concentration of 0.2 to 2%, and hulupones less than 0.5% by weight.

1.2.2. Hard resins: xanthohumol and derivatives

Compared with the soft resins, the hard resins of hops are composed of more polar compounds, reflecting their insolubility in hexane. They contain a complex mixture of polyphenols, namely, proanthocyanidins, flavonol glycosides, prenylchalcones, with xanthohumol and desmethylxanthohumol being the most important molecules of the group, and also prenylflavanones, as isoxanthohumol, 6-prenylnaringenin and 8-prenylnaringenin (Figure 1.4).

xanthohumol desmethylxanthohumol

isoxanthohumol 8-prenylnaringenin 6-prenylnaringenin

Figure 1.4. Chemical structures of prenylflavonoids xanthohumol, isoxanthohumol, desmethyllxanthohumol, 6-prenylnaringenin, and 8-prenylnaringenin.

These compounds are recognized as presenting beneficial health properties, in particular antioxidant, anti-inflammatory, anticancer, immunomodulatory and antimicrobial (50, 51). Prenylchalcones, particularly, received much attention in the last years due to its potent chemopreventive properties. Xanthohumol is a promising anticancer agent, whereas 8-prenylnaringenin, the demethylated derivative of isoxanthohumol, has been the target of several research works due to its potent phytoestrogenic activity (52-54). Dry hops may contain up to 1% dry of xanthohumol whilst 8-prenylnaringenin is usually present in concentrations lower than 10 mg per 100 g.

Although beer bitterness is traditionally attributed to the soft resin compounds, some hard resin molecules had been recently associated as contributors to the bitter taste of beer. This is the case for xanthohumol and its isomerization product isoxanthohumol, as well as, desmethylxanthohumol and isomerization products of 8-prenylnaringenin and 6-prenylnaringenin, by coactivation of some of the tongue bitter taste receptors (55). In fact, it was reported that brewing beer using the isolated hop hard resins fraction was found to give beverages a strong and pleasant bitter character, indicating the presence of additional valuable bitter compounds in the hard resin (56).

1.2.3. Volatile fraction

The hop aroma is another desirable feature of beer and is directly associated with the presence of essential oils, formed by a complex group of volatile compounds. Around 440 molecules have already been identified. Nevertheless, more recent analysis using comprehensive multidimensional gas chromatography (GC × GC) with flame ionization detection suggested that more than one thousand compounds can be found in this fraction (2). The main constituents include a highly diverse group of esters and terpenes. The monoterpene β-myrcene, together with the sesquiterpenes α-humulene and β-caryophyllene are the predominant components in mass terms. Although in smaller amounts, other chemical groups are also present, such as aldehydes, aliphatic hydrocarbons, carboxylic acids, esters, furans, higher alcohols, ketones, phenols and sulphur compounds (32, 57-59).

The noble hop aromas are typically classified by sensory analysis and olfactometry trials according to specific descriptors, which follow within the categories of citrus, fruity, floral, spicy, resinous (woody aromatic), herbal and cream caramel or sweet-like (Table 1.1).

Table 1.1. Common aroma descriptors and odour qualities attributed to hops in sensorial and olfactometry analyses.

Odour quality		Aroma descriptors
Citrus		grapefruit, orange, lime, lemon, bergamot, lemongrass, ginger, tangerine
Fruity	green fruits	pear, quince, apple, gooseberry, wine white grape
	red berries	cassis, blueberry, raspberry, blackberry, strawberry, cranberry, red currant, black currant
	sweet fruits	banana, watermelon, honeydew melon, peach, apricot, passion fruit, lychee, dried fruit, plum, pineapple, cherry, kiwi, mango, guava
Floral		elderflower, camomile blossom, apple blossom, lily, lily of the valley, lilac, jasmine, rose, geranium, carnation, lavender
Spicy		pepper, chilli, curry, juniper, aniseed, liquorice, fennel seed, clove, cinnamon, gingerbread, coriander seed, nutmeg
Resinous (woody aromatic)		tobacco, cognac, barrique, leather, tonka, woodruff, incense, myrrh, resin, cedar, pine, earth
Herbal	herbal	lovage, marjoram, tarragon, dill, thyme, rosemary, basil, parsley, fennel, coriander, sage, tea, green tea, black tea, mate tea
	menthol	mint, melissa, camphor, balm, wine yeast
	green grassy	grass, tomato leaves, green pepper, nettle, hay, cucumber
	vegetal	celery root, celery stock, leek, onion, artichoke, garlic, wild garlic
Cream caramel (sweet-like)		butter, chocolate, yoghurt, honey, cream, caramel, toffee, coffee, tonka bean, vanilla

Over the years the chemical characterization and quantification of the compounds associated with aroma attributes has been performed. Monoterpenes are related with citric, spicy, resinous and herbaceous categories; the sesquiterpenes are associated with spices and woods, the esters with fruits and sweets-like odours, ketones with floral notes and aldehydes with grassy/ green attributes (31, 60-62). This complexity is tentatively resumed in Figure 1.5, which gives an overview of the main groups of compounds forming the hops aroma, the principal chemical components and the odours attributed to each molecule alone.

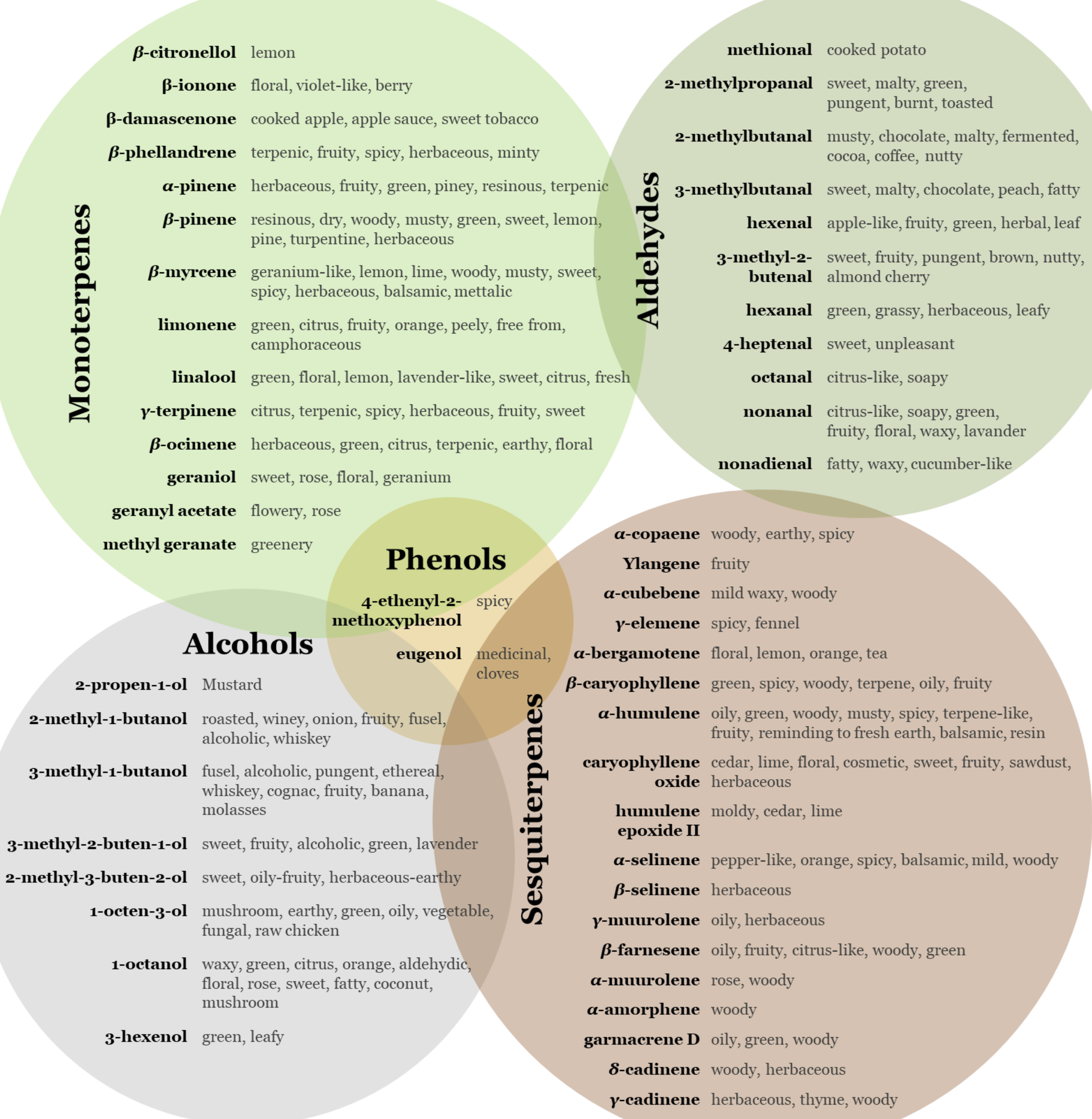


Figure 1.5. Main aromatic compounds of hops and the corresponding odour description.

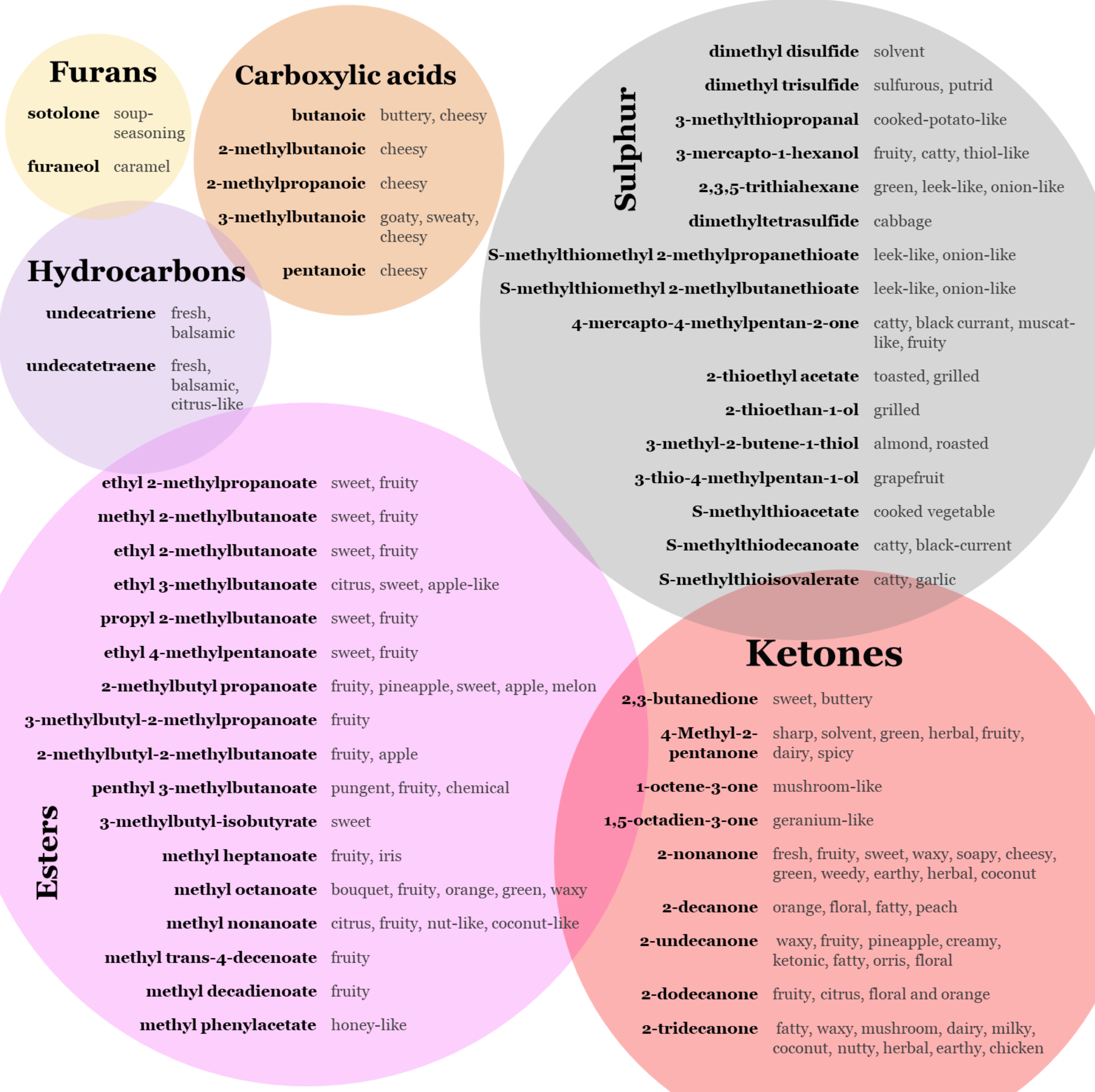


Figure 1.5. Main aromatic compounds of hops and the corresponding odour description (continued).

Inside the terpenes group, oxygenated monoterpenoids (citrus and floral), as linalool, citronellol and geraniol, as well as, oxygenated sesquiterpenoids, caryophyllene and humulene derived epoxides (spices and herbaceous) received special attention due to their solubility and stability. Although their amounts in hops are low they represent the most relevant compounds that remain in the final beers, especially when hops are introduced in the boiling kettle stage of brewing, which corresponds to the more traditional approach of hops usage (63-65). Once they are present in different concentrations among the hop varieties, it is not clear if these derivatives are mainly formed by oxidation during hop storage or by chemical transformation during wort-boiling (66).

Late hop addition in the last 5-10 minutes of boiling enhances beer aroma, but even this technique leads to losses of some aromatic oils that evaporate rapidly in the boil. Greater extraction and retention of volatile compounds in beer is observed when hops are added at the cold stages of brewing (67, 68), which affects significantly the aroma of those beers. Dry hopping involves adding hops to the fermenter or after fermentation. This technique adds aromatic compounds that are normally lost in the boiling process, but it does not increase beer bitterness. Dry hops are allowed to soak in the finished beer during several days or even several weeks. The result is a great increase of hoppy aroma.

Nevertheless, due to the high variation among varieties and the intrinsic complexity of the hop oil, as well as, the diversity of brewing processes, together with the lack of standardization of analytical methods for the analysis of individual volatile compounds, it was not possible until know identify all the individual hop components that impart the noble hop aroma. Moreover, the exact contribution of each compound to the specific sensorial quality of beers is not known, which constitutes another hot subject on hops and beer research.

1.2.4. Hop varieties

Hop is usually propagated via vegetative cuttings, i.e. stem/leaf and most commonly rhizomes, following a clonal propagation system for varietal distribution. The female inflorescence or cone is the product of interest for the brewing industry. Male plants are only required for breeding purposes, since male pollen is needed to fertilize de female inflorescence when the search for new plants derived from natural genetic recombination is pretended. Female plants produce cones without pollination, therefore in hop fields there are only female plants (10). In fact, the presence of male plants in the surroundings is even undesired as their pollen can be dispersed via air flow and pollinate females, which will produce seeded inflorescences, not desired by brewers. The organoleptic features of the resulting hop will be affected due to the seed compounds themselves. Hops present a high varietal diversity, as observed for most of the cultivated plant species.

Hundreds of hop varieties are known nowadays, 270 varieties (or brand names) were listed in the most actual revision of the International Hop Growers' Convention (69). Some hops received special attention. Typical hops, like the English Goldings (GOL) and Fuggle (FUG), are named as "noble hops", but some varieties from Hallertau, Spalt, and Hersbruck regions of Germany and the traditional Saaz (SAZ), growing in the Czech Republic, become also famous and have been largely used. Notwithstanding, due to the actual changes in brewery practices, there has been an intensive search for new varieties of the plant, dubbed "Green Gold". Therefore, a large number of new varieties have been developed,

characterized, and increasingly commercialized. This is the case of the German hops Perle (PER) and Herkules (HKS), the American Centennial (CEN), Cascade (CAS), Amarillo® (VG1), Simcoe® (SIM), Citra® (CIT), and as well as new varieties out of the axis England / Germany / US, *e.g*. Nelson Sauvin™ (NSN) from New Zealand. Figure 1.6 presents the main hop commercial varieties that are available in the market in the form of pellets, taking in consideration its importance throughout the history or its current or potential market share. Varieties are distributed in a bitter scale (% of α-acids), but information regarding their aroma profile, country of origin and the most common uses are also added. The selected hops merged, account for more than 80% of the current world harvest.

Figure 1.6. Hop varieties displayed according to the average of α-acids %, including information regarding their aroma profile, country of origin and the purposes of most common uses. Green, red and blue represent aroma, bitter and dual purpose hops, respectively.

Besides the sensorial aspects, hops varieties also differ botanically (since different size and forms of leaves and cones can be observed) and agronomically, due to variation in soils adaptation, yields, time of maturity, and resistance against pests and diseases. Qualitative and quantitative differences are observed on chemical composition of different hop varieties. Plants may also present relevant differences when the same variety is cultivated in different regions that have different soil and climate conditions. Moreover, even when planted in the same edaphoclimatic conditions, the hops may present different composition in diverse years of production due to climatic variations. Thus, it is very important, and a common practice after each crop, to perform the chemical characterization of the harvest.

1.3. New trends of hop use

1.3.1. The craft brewery movement

The historical worldwide restrictions on alcohol consumption in the end of the century XIX and beginning of the century XX, added by the two big world wars, resulted in a drastic drop in beer companies based in traditional producing countries. For example, in Belgium, breweries decreased from 3,223 before the first war to 755 in 1946; in US, breweries decreased from 2,300 in 1880 to 160 at the beginning of World War II and to 60 at the early 1960s; whereas in the United Kingdom, there were 6,447 breweries in 1900, which reduced to 885 in 1939 and to 358 in 1960 (70).

On the one hand, after the Second World War, a climate of rebirth emerged in all sectors of society, the world was affected with globalization of markets (remembering that internet began in 1955 and opened of the network to commercial interests in 1988), promoting the gigantism of companies and products massification. In the beer market, the brewers master became hostage of the marketing departments, product changes were directed expressly to increase sales volume and profits, resulting on production, in large scale, of the called Standard American and International Lager styles with low costs of production, simplicity in flavours, and designed to appeal to mass-market drinkers (71). Moreover, there is a growing concern its environmental sustainability, which induced a change in consumer's point of view, becoming more and more demanding. Nowadays, consumers look for products that are healthy, ecologically sustainable and present good quality, paying attention to novelties and sophistications. This is accompanied by the proliferation of small producers offering diversification and experimentations. In the 70's, the brew sector faced the renaissance of European and North American brewery. Standing out the movement Campaign for Real Ale (CAMRA, begin in 1971), and the US development with a big wave of

microbreweries (1976 was founded The New Albion Brewing Company, considered the first US microbrewery of the modern times) and homebrewers. Rescuing creativity and dynamism from a dormant tradition, different ingredients and processes have been used by brewers to produce new styles of beers and different variations of traditional styles.

1.3.2. Forms of hop usage

1.3.2.1. Hops products

Hops are commercialized in cones which can be in pure state after the harvest or dried and pressed. However, due to the logistic of transporting and storage, there are smaller and more stable forms, such as hop pellets, produced from dried milled and pressed inflorescence with vegetative content and extracts derived products (Table 1.2).

Table 1.2. Types of hop products commercialized nowadays and respective preparation process.

Hop products	Preparation process
Fresh cones	pure state
Dried cones	dried and pressed
Pellets	dried, ground and pressed
Extracts	extracted from pellets or cones by supercritical CO_2 extraction, nitrogen-rich atmosphere, ethanol or distillation methods

Big breweries produce mass-market products and generally use hops only to reach bitterness and prefer enriched α-acids obtained from supercritical CO_2 extraction. On the other hand, the raw materials used by small breweries are closer to the natural forms, thus provide a higher complexity of flavour to the final product, when compared with hop products derived from extraction, mainly because part of the volatile fraction is lost during the extraction methodologies (72, 73).

1.3.2.2. Brewing production

Hops are mainly used to produce beer. They can be added at different stages of beer production (36), namely: (i) mashing, mash preparation from milled barley malts and water (this process includes temperatures from 50 to 78 ºC); (ii) lautering (mash out,

recirculation, and sparging with hot water), in which mash is separated into clear liquid wort and residual grains; (iii) boiling at 100 ºC, usually from 60 to 90 min, phase to sterilize the wort and addition of hops for bitterness (at the beginning) and flavour reasons (at the end); (iv) whirlpool, for removal the trub, a mixture of spent hops, heavy fats, and coagulated proteins (v) cooling, until appropriated temperature to inoculate the yeast; (vi) fermenting, normally at temperatures between 6 to 12 ºC to Lagers, and 15 to 25 ºC to Ales beer styles; (vii) maturing or lagering (or still, second fermentation), at lower temperatures, with the purpose of removing undesirable flavorous by-products of the called "green beer" (74); (viii) filtering, used in most of beer styles, for clarify; and (ix) packaging, frequently in bottles, barrels, kegs, and cans.

1.3.2.3. Dry-hopping

Distinct beer flavour can be imparted by different hops varieties, but also by the time of hop addition, the amount added, the brewing method and the beer matrix. Moreover, the old practice of introducing the hops only during the wort boiling is not very efficient to retain hop flavour, since volatile components are evaporated or chemically changed. The dry hopping techniques that add hops after the boiling phase of the brewing process, at cold stages are increasingly been used by the breweries (1). Therefore, many dry-hopping techniques has been used varying hops product forms, quantities, time of contact, temperature, and moment of addition (during the fermentation, or after, in maturation stages, with or without yeast presence). All those factors influence the transference of flavours from hops to beers (15-19).

A confirmation of the success of the dry-hopping techniques is the large acceptability and worldwide consumption of the beer style American India Pale Ale (American IPA) (75). In this kind of beer the US varieties of hops are the main components, providing bitterness and floral, fruity, sulphur, citrus and resinous aromas and flavours (71, 76).

In general, the craft brewery movement provided a new market trend for hops. Since microbreweries use about 10 times more quantities of hops per litre of beer, and much bigger spectrum of cultivars when compared with the traditional big beer companies (72).

1.4. Market

According to data from beer sector, hops plantation has been a profitable investment and a factor of regional development, and so, increasingly, countries have invested in this sector. Beer is the alcoholic beverage most consumed in the world. Interrupting growth since 1998,

the year of 2014 was the first that the beer market registered a drop of word production, down of 0.5% in relation with 2013, when 1.963 billion hectoliters of beers were produced, since then, three drops, 1.961 billion hL in 2015, and 1.951 billion hL in 2016. In 2017, a small increase to 1.952 billion hL (77).

Additionally, it is important to emphasize that the craft beer market, managed by small breweries, has been growing strongly over the past few years. In the United States, craft breweries are defined as small, with a maximal annual production of 6 million barrels (approximate 715.4 million liters), and independent, more than 75% must be owned or controlled (or equivalent economic interest) by itself, since 2017 microbreweries carry a seal certified by the Brewers Association. In 2013, there were 2,898 breweries in this condition, most recent data appointed 7,346 microbreweries (78). Representing 13.2% in volume of production of US beer market, and a financial turnover of US$ 27.6 billion, accounting for more than 24% of the total dollar sales (79). In the UK, from 898 microbreweries in 2013, last report amounted to 2,378 microbreweries in a total of 2,430 breweries companies (80). Data from the same years indicate a growth from 659 to 824 craft breweries in Germany and, even in countries with strong wine of expression, and little beer expression until recently, such as France and Italy, more than doubled the number of breweries in the last six years, ending the year of 2017, with 1,000 and 693 microbreweries, respectively. Switzerland had 313 microbreweries in 2013, having 818 in 2017, in Spain another big expansion, from 70 to 502 microbreweries in the same period. In Portugal, the first craft brewery appeared in 2011, and in 2017 there were 115 breweries (80). Also in Brazil there is a notable growth the number of breweries, that grew from 266 in 2010 to 679 in 2017, values attributed to the craft beer sector, although there is no specific definition for craft/ microbreweries in the country (81).

After five years of decrease, from 57,297 ha in 2008 to 46,246 ha in 2013, there is increases of the global area of hop cultivation, and, in 2017, the amount harvested was 58,739 ha, 27% higher than the harvest of 2013 (3), although the total production of beer remained stable in the same period (77). Which could be a concern for hops market. These data corroborate the increase of hops ratio per liter of beer, typical characteristic of craft brew sector (Hintermeier, 2016). In 2018, there was a new worldwide rise of hops, since around 60,300 ha were put in the ground (82).

1.4.1. World production

There is a serial of governmental and no-governmental organizations that give support to the hop growers, like the private company Joh. Barth & Sohn GmbH & Co KG, and the association of hops growers Hopfenring e.V., the Association of German Hop Growers

(*Verband Deutscher Hopfenpflanzer e. V.*), the International Hop Growers' Convention (IHGC), the Slovenian Institute of Hop Research and Brewing (*Inštitut za hmeljarstvo in pivovarstvo Slovenije*, IHPS), the National Agricultural Statistics Service (NASS) from United States Department of Agriculture (USDA), and the Hop Growers Union of the Czech Republic (*Svaz pěstitelů chmele České republiky*), and the British Hop Association. The data summarized in this section is a compilation of the last reports.

Over the years, most of hops acreage were in direction of to produce α-acids. However, the growth of craft brew sector, and the development of new flavoured beers, had essential influence in the balance of the harvest for aroma and alpha varieties. Since 2011, the acreage of aroma hops increased more than 71%. In 2014 occurred the inversion of the situation, since then having more production of aroma hops. In 2018, the acreage was 40,124 ha of aroma hops. Much higher than the 18,989 ha of alpha varieties. Being produced 68,768 ton and 48,865 ton of hops with aroma and alpha purpose, respectively. It was also noticed the acreage of 2,344 ha of new (aroma and alpha) hops varieties (83).

More than 76% of world crops are in the two main hop growing countries, Germany and US. During many years Germany was isolated as the main hops producing country. Confronted a sequence of decline of hop acreage between 2009 and 2013, however the tendency was inverted in 2014, with sequence increases of the harvest (3, 84). From the acreage of 2018, was produced 41,727 ton of hops (85). Hallertau, in Bayern, was the main producing region, since had cultivated about 87.5% of total production. In addition, basically three other regions completed the hops harvested: Elbe-Saale (6%), Tettnang (5%), Spalt (1.5%). After a long period of investment, studies, breeding's and research, the United States of America have produced a great number of hop varieties and in 2015 overcame Germany and became the country with the highest number of hectares planted of hops in the world (Figure 1.7). There are three main regions of hop-growing in the country, in 2018, Washington represented about 71.2% of US acreage, Idaho 14.8%, and Oregon 14% (86).

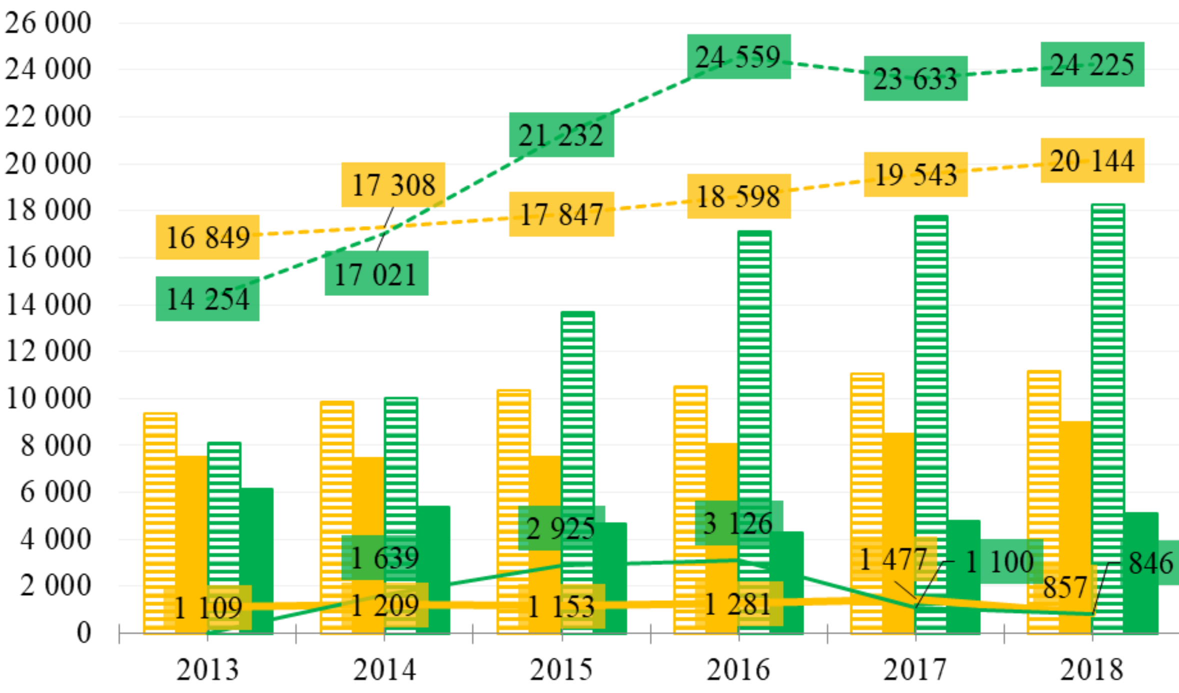


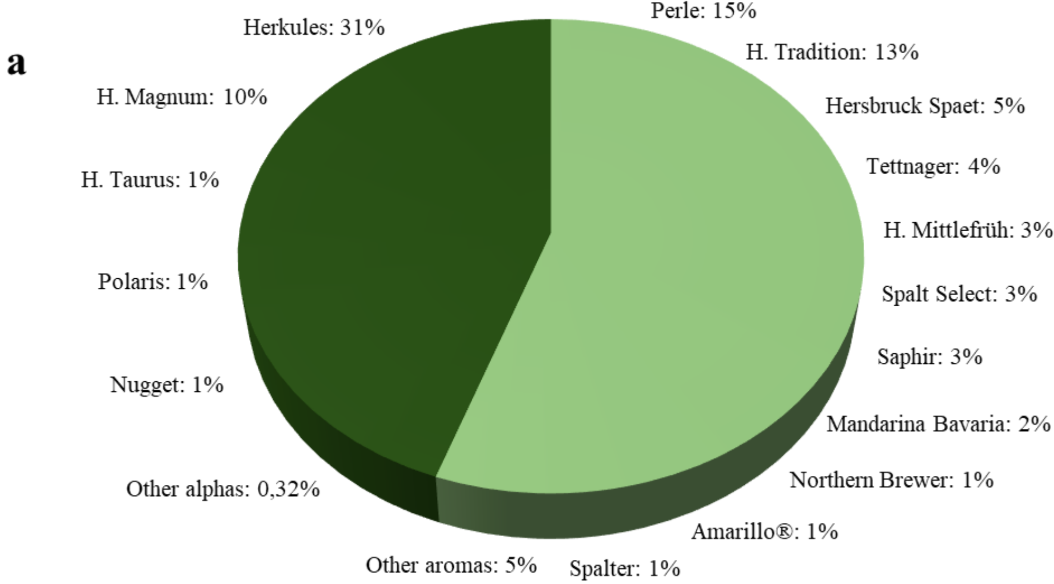
Figure 1.7: Comparison of last six years of acreage, in hectares, and production, in tons, of hops in Germany (yellow) and US (green). Bars represent aroma (with stripes) and alpha (without stripes) purpose varieties. Continue and dashed lines represent new varieties and total acreages, respectively.

Historically, both countries present good yields in their crops (87). In the period from 2013 to 2018 the yields were, in average, around 1.9 and 2.1 ton per hectare in Germany and US, respectively. In Germany, there was a higher variation, from 1.5 (2015) to 2.2 ton/ ha (2014), in US, the yield was more stable, varying between 1.9 (2016) and 2.2 ton/ ha (2013 and 2017).

In 2018, around 55% (11,168 ha) of Germany acreage was for production of around 20,000 ton of aroma varieties, predominantly Perle (PER) and Hallertauer Tradition (HTR), also Hersbrucker Spät (HEB), Tettnanger (TET), Hallertauer Mittlefrüh (HAL), Spalt Select (SSE), Saphir (SIR), and Mandarina Bavaria (MBA) (82). About 45% (8,976 ha) was for production of around 21,700 ton of alpha varieties, resulting in 3,920 ton of α-acids production. The alpha varieties were principally Herkules (HKS) and Hallertauer Magnum (HMG), Hallertauer Taurus (HTU), Polaris (PLA), and Nugget (NUG) (Figure 1.8a).

In accordance with the craft brew sector, over the past six years the US producers emphasized in the production of aroma varieties (Figure 1.7). In this period, the acreage of aroma varieties grown up more than two times, from 8,092 ha in 2013 to 18,257 ha in 2018, which represents around 75% of the total US hop acreage, producing 33,292 ton of aroma purpose hops, including Citra® (CIT), Cascade (CAS), Centennial (CEN), Simcoe® (SIM), Chinook (CHI), Mosaic™ (MOS), Amarillo® (VG1), Pahto™ (PAH), Willamette (WIL),

Ekuanot™ (EKU), Crystal (CRY), College Cluster (CLU), Azacca™ (AZA), Eureka™ (EUE), El Dorado™ (ELD), Palisade® (PAL), Galena (GAL), Mount Hood (MTH), and Comet (COM). In other hand, 5,122 ha were to produce 15,292 ton of alpha varieties, resulting in 5,223 ton of α-acids. The predominant varieties include Columbus (CBS), Tomahawk® (TOM), and Zeus (ZEU), also include, Summit™(SUM), NUG, Apollo™ (APO), Super Galena™ (SGA), and Bravo™ (BRO) (Figure 1.8b).



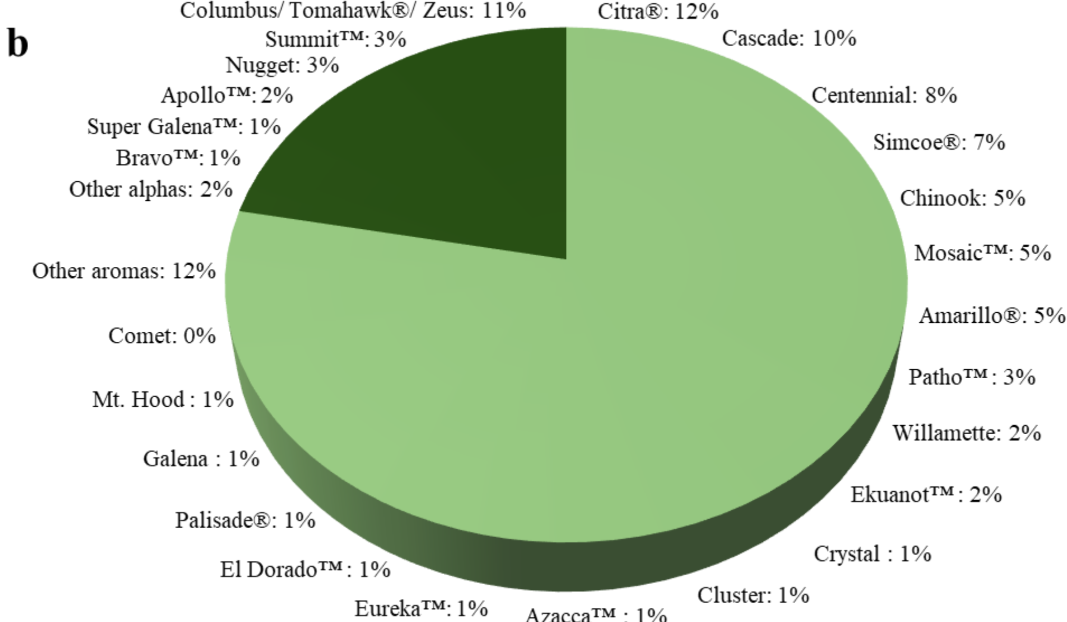


Figure 1.8: Acreage (%) for the main hops varieties in 2018 in (a) Germany and (b) US. Light and dark green colours represent aroma and alpha purposes of varieties, respectively.

The same tendency in harvest balance of aroma and alpha purpose varieties can be observed in the other producer countries. Adding to Germany and US, seven countries complete over 95% of worldwide hops production. In Czech Republic, Slovenia, England, Australia and New Zealand, the production of aroma is much higher than alpha varieties. Opposite of China, Poland, and Spain, where the focus is to produce alpha varieties. Total, aroma and alpha acreages, and the yields of these and other producer countries are showed in Figure 1.9.

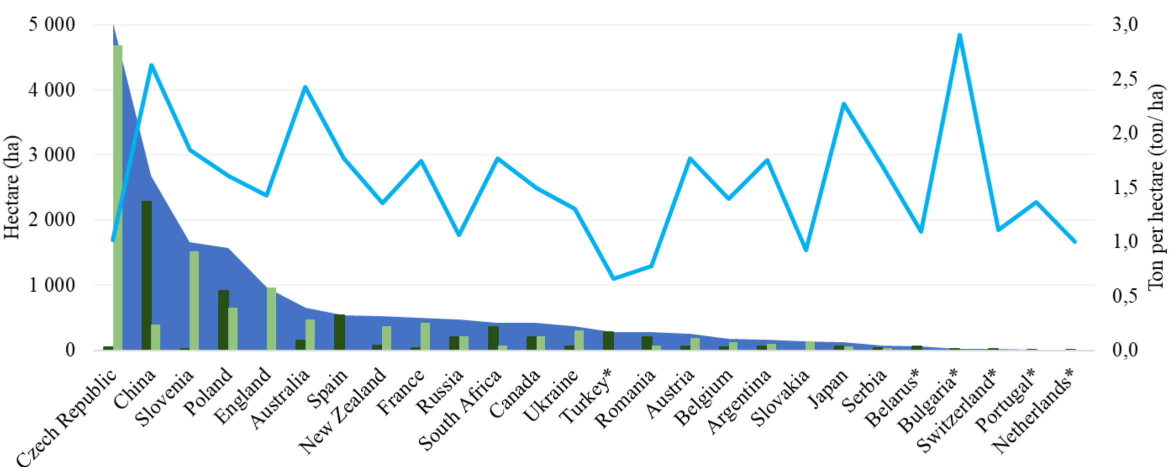


Figure 1.9: World hops acreage (ha). Bars represent aroma (dark) and alpha (light) purpose varieties. Area and line represent total acreage (ha) and yields (ton/ ha), respectively. Data from crops of 2018 and 2017*.

Czech Republic is the third largest producer of hops, and is the most important producer of SAZ, famous aroma variety largely used in the Lager Beer Style that dominates the world beer market share. The country has already planted more than 17,000 ha at the begin of the 21th century, however it has presented a decline in acreage over the years (88). In 2013, the country harvested 4,319 ha, nevertheless in 2014 the growing was reassumed, and in 2018, 5,020 ha were harvested, producing 5,100 ton of hops. Predominantly aroma variety SAZ, about 87% (4,349 ha), and other varieties, such as, Sladek (SLD), Premiant (PRE), Saaz Late (SAL), and Agnus (AGN), the only alpha variety (82, 89).

The statistics on acreage and production volume in China are based on estimates in the size of the Chinese hop-growing regions of 24 farms in two regions, Xinjiang and Gansu (3), which, in 2018, together produced 7,044 ton of hops in 2,683 ha, keeping China in the top yields list, and in fourth colocation on the rank of word production. The main production

has been of the alpha variety Tsingtao Flower (QID). Aroma SA-1 (SA1), Kirin Flower (JTY) and Marco Polo (MCP) are other varieties cropped in in the country (82).

In Slovenia, producers are particularly investing in the aroma varieties, in 2018 it was 1,526 ha (3,018 ton) for aroma and 21 ha (60 ton) for alpha purpose, with great success on the fine aroma varieties Styrian Golding Celeia (SGC), Super Styrian Aurora (SSA), Styrian Savinjski Golding (SSG), and Styrian Golding Bobek (SGB). In Poland, the most harvested was of alpha hops, 918 ha (1,700 ton) from a total of 1576 ha (2,530 ton). Besides HMG, attention to the Polish high alpha varieties Marynka (MAR) and Magnat (MGN). Lubelski (LUB) and Sybilla (SYB) are the most harvested aroma varieties (82).

Hops start to be breeding, in Kent, England, and, until nowadays, the country have an important breeding program in the beginning of the 20th century, being over this year, together with Germany and US, important for the development of new varieties (90). Such as all other countries producers, England has resumed recently the increase of hops production. The year of 2016 marked the end of four years of decline of hops crop in England, since then the production is increasing. On the ground old aroma varieties, well known to make ingredients to produce historical English Ale Beer Styles, mainly Golding (GOL), East Kent Golding (EKG), First Gold (FGO), Fuggle (FUG), Progress (PRO), Wye Challenger (CHA), Sovereign (SOV), Bramling Cross (BRX), and Whitbread Golding (WGV) in addition to other aroma varieties, dominated the plantations with more than 80% of 966 ha (1,377 ton). Pilgrim (PGM), Wye Target (TRG), Admiral (ADM), and Northdown (NOR) were the alpha varieties more cropped (3, 82, 91).

In Oceania the acreage 2018 was 652 ha (1,582 ton) and 531 ha (722 ton) in Australia and New Zealand, respectively. Both with focus on aroma varieties 479 ha (1,228 ton) in Australia, and 371 ha (600 ton) in New Zealand (82). Galaxy™ (GXY), Super Pride (SUP), Vic Secret™ (VIS), Pride of Ringwood (POR), Ella™ (ELL), Enigma™ (ENI), and Topaz™ (TOP) are the main varieties in Australia (3). New Zealand cultivation is dominated by Nelson Sauvin (NSN) and Motueka (MKA) (92). Wakatu (WKT), Taiheke™ (CAS), Pacifica, Rakau (RKU), Green Bullet (GBU), Wai-iti (WTI), Pacific Gem (PGE), Pacific Jade (PJA), Dr Rudi (DRU), Kohatu (KHT), Waimea (WAI), Southern Cross (SOX), Riwaka (RWA), Moutere (MTE), and Stickelbract (SBR) complete the New Zealand varieties, with emphasis for the program of organic hops production (92).

There is no commercial hops variety originated from Spain. In 2018, it was 537 ha, producing 960 ton, all for alpha purpose (82). For long time, the crops have been, mainly of NUG, CBS and HMG varieties, however, after investment in the sector, since 2014, new varieties, such as American EUE and French Apollo™ (APO) has been cultivated in direction of the renovation of the sector in the country (93).

The sum of other producer countries does not reach 5% of the total world hop volume. Alsace dominates the hop cultures in France, having a small production also in North of the country (3). Investment have been done in order to development of breeding and organic production programs (94). Aroma hops are predominant, in 2018 the total acreage was 496 ha (864 ton), being 424 ha (775 ton) of aroma, including native varieties Strisselspalter (FSP), Aramis (ARS), Triskel (TKL), Barbe-Rouge (BBR), and Mistral (MTL) varieties (82). Russia harvested 470 ha, producing 500 ton of hops, in equal proportion of aroma and alpha varieties, In South Africa, the alpha varieties predominated the 2018 acreage, it was 354 ha (677 ton) in a total of 427 ha (754 ton).

In Portugal the hops acreage is reduced only to 12 ha of NUG variety around of the city of Bragança, in the Northeast region of the country, which produced 16.4 ton of hops in 2018, representing only 5% of the country's need. However, there is a great interest of the region to become again a strong producer of hops, as it was in past decades. The peak of hop production was in 1976, 205.8 ha were cultivated which produced 438.1 ton of hops, which generated self-sufficiency in Portugal producing 100% of the needs for breweries and still left to export. Recent history shows that the Northern of Portugal has ecological potential to compete with productivity and quality in the international market among the largest world producers of hops. In this sense, actions have been made, to sensitize farmers, entrepreneurs and authorities to the need of restructuring of the hop sector. There are some initiatives in development, and projects for the selection of new cultivars adapted to the region, demonstrating that there are conditions to attract new producers of hops (73).

1.4.2. Hops business

The growth of craft beer movement promoted that the aroma varieties are the most valued in the market, however the market prices of high alpha varieties continues rising, which can be justified by the fact that the volume of alpha varieties produced has not been sufficient for the demand. The influence of microbreweries it is again unnoticed, once the characteristic of this brew sector favours more flavoured beers, more aromatic but also more bitter than the beer produced by the big companies. Therefore, even in absence of growth of beer production, in the last years the rate of alpha-acids per hectolitre of beer raised from 4.68 g (in 2013) to 5.71 g (in 2018), promoting the demand of alpha-acids, however, the world supply along the years, provided mainly German high alpha-acids hop has been insufficient to compensate US hop plantation changes (3).

The annual high demands of hops, from all kind of varieties, has increased the prices, and makes the plant, more and more, a profitable business, being common to have forward contracts between the hop growers and purchasers. In Germany and in other countries of

EU normally the trade is with hops dealers, whereas in US, usually the activity is directly with the brewers. In general, aroma varieties have more value than bitter, and the American varieties presenting higher prices (3, 95). Another difference in the two biggest markets, is that in EU the contracts have long periods of extension, normally reaching more than five years forward, and growers trade practically the full production, having short marge for spot market, only if there is a good crop, with a high yield. While in US, normally contracts are not so long and there are more hops for spot market.

1.5. Updates in hops research

1.5.1. Analytical methods for hops and beers

The American Society of Brewing Chemists (ASBC) and the European Brewery Convention (EBC) are two main committees that standardize the analytic methods used during the brewing process (namely, wort analysis and microbiological control), to control beer quality, as well as the analytic methods to be applied on analysis of raw materials, including hops, water, barley and yeast.

One of the most important parameters to be evaluated in hops is the percentage of bitter acids. Since the early 1980s, the analysis of specific acids has been increasingly performed by high-performance liquid chromatography (HPLC), although spectrophotometric and lead conductance titration methods are also largely used (titration based on the reaction of their ionized forms with lead salts). Both committees, ASBC and EBC, describe HPLC as the International Method to provide α- and β-acids concentrations by using the International Calibration Extract (ICE). This is a mixture with known concentrations of α and β-acids, provided by the ASBC and the EBC, together with the Institute and Guild of Brewing and the Brewery Convention of Japan in collaboration with the hop industry. Thus the individual concentrations of some compounds that are chromatographically separated can be measured, as is the case of *co*humulone and *co*lupulone (96, 97).

Notwithstanding, due to the lack of specific equipment in most of the laboratories used for hop analysis, the most common method for bitter acids analysis is still by ultra-violet (UV) spectrometry (98). Bitter acids are determined after extraction with an organic solvent. The ASBC method indicates extraction with toluene and further treatment with methanol for separation of α-acids and an alkaline methanol extraction for β-acids. However, the method suffers interferences from the oxidation products of α- and β-acids, thus, it is proposed to measure the absorbance at the wavelengths of 355, 325 and 275 nm, and undertake mathematical corrections to reduce errors.

Beer bitterness is associated with the international bittering unit (IBU), calculated taking into consideration the percentage of α-acids added and time of boiling (isomerization). Iso-α-acids are also a parameter used to beer bitterness, being measured by spectrometry or HPLC (99, 100).

The Hop Storage Index (HSI) is another important parameter for brewers and is used to determine hop oxidation levels that can occur during hop storage. HSI increases when inadequate storage conditions are applied. The spectrophotometric method is also used to evaluate HSI, which is calculated throughout the progressive increase in the ratio of absorbance at 275 to 325 nm in the alkaline methanol extract (101).

Although less used in hops and beer characterization, the measurement of total polyphenols content (TPC) is also important, considering its technological impact and health effects due to their bioactive properties. TPC is generally determined by spectrophotometry, in both hops and beer (102, 103). However, HPLC is the preferred method to quantify individual flavonoid compounds of hops and also to monitor their progress in hop products and beers. Several methodologies have been developed coupling HPLC, UV, diode array detection (DAD) and mass spectrometry (MS/MS) (104-106).

The composition of hops essential oils has been extensively studied since Chapman's early classical chemical techniques (107-109). Advances in gas chromatography, including high-resolution capillary columns, and the coupling mass spectrometry detector (GC-MS), first reported in 1965, by Buttery, provided more detailed information about the profile (110). Chromatographic fingerprint analysis of hop volatile fraction by gas chromatography and mass spectrometry can be a useful tool for varieties authentication and for quality evaluation. Over 400 compounds can be separated and estimated in one single run by GC-MS analysis of hops essential oils, which enables a comparative study of different plants by chromatographic profiling or quantification (111, 112). The steam distillation method is commonly used to obtain hop essential oil, however, it requires a relatively large amount of sample (50-100 g) and is time consuming (113, 114). The use of headspace (HS) sampling allows the analysis of a high number of samples in a relative short period of time and is easily automated. Different HS methods can be used, involving minimal sample preparation and rapid enrichment of volatile or semi-volatile compounds during the HS analysis. Several HS techniques were used for the determination of hops volatile compounds (62, 115, 116). HS methods include dynamic sampling method such as in-tube extraction (ITEX) or static methods, namely solid phase microextraction (SPME) (117). Both used to study of volatile hops-derived compounds in beers (118-120).

Besides analytical identification and quantification of hop and beer compounds, human sensory perceptions are an important tool to characterize hops and its influence in beer flavours. Gas chromatography olfactometry (GC-O), with trained human assessors, has

been widely used to identify and characterize hop compounds that can be detected by human noise, usually called odour-active compounds (60-62, 121). The same for hop-derived compounds in beers (64, 122). Sensory analysis with trained, or semi-trained panellists of hops and beers point specific aroma attributes (13, 123, 124), contributing to the better knowledge of the relationship of volatile fraction with flavour characteristics (1, 14, 65).

1.5.2. Bioactivity

Hops has been used as a medicinal plant for more than 2000 years against leprosy, constipation, foot odour, and to purify the blood (125). The biological activity of hop bitter acids was first described in the 1990s (126), by determining theirs antioxidant activity, since then the bioactivity of several hop compounds present in soft and hard resins is, thus, well known and widely studied, given raise to some good revision works on the subject (39, 127). Beneficial effects on the human health are mainly related with the antioxidant (polyphenols and bitter acids), antimicrobial (bitter acids, polyphenols and essential oils) and sedative (resins and oil) effects, as well as, the prevention of neurodegenerative (naringenin, xanthohumol) and cardiovascular disorders (polyphenols) and to estrogenic (8-prenylnaringenin), antiproliferative (xanthohumol, kaempferol, 8-prenylnaringenin), antiangiogenic (xanthohumol and isoxanthohumol), and antinflamatory (xanthohumol and 8-prenylnaringenin) activities (39, 51). Notwithstanding, in the last few years, some newly discovered molecules have been described and their effects on health studied as promising phytotherapeutic agents reaffirming the high potential of the plant as a source of new molecules.

One of those classes of new compounds are the humulinones (Figure 1.3), naturally formed by the oxidation of α-acids in the hop cone, which occurs promptly during storage. Hops with higher HSI (hop storage index) are rich in humulinones (128). Biter acids oxidation products had already been obtained by chemical oxidation of isolated α- or β-acids but their occurrence in hop cones have remained uncertain until recently (129). Chemically, humulinones are hydroxylated iso-α-acids, thus, more polar and soluble in beer, and were reported to be between 0.2 and 0.5% by weight, in several hop pellets and whole leaf, whereas hop extracts contain none. Although with lesser bittering intensity than iso-α-acids they can contribute to the beer overall bitterness when used in dry hopping since over 87% of all the humulinone molecules will dissolve in the beer after 2-3 days of extraction (128). Besides its importance in bitterness, humulinones probably present high biological activity due to its similarity with the molecule of α-acids, notwithstanding, their

potential health benefits are not well understood (130). The most important metabolites of α-acids were identified as humulinones and hulupones. Interestingly, the phase I metabolites are highly similar to the oxidative degradation products in hops and beer (131). These findings show a first insight into the metabolites of hop-derived bitter acids and may have practical implications in the bioavailability of these compounds, following ingestion of hop-based products.

Hulupones are formed from the oxidation of β-acids (Figure 1.3), in the hop cone as well as during boiling, however in a small amounts, and also contribute to the bitter taste (132). Hulupones are also promising bioactive substances as they derive from β-acids to which several physiological effects had been attributed, particularly, their antiproliferative action in cancer cells (51).

Other putative α-and/ or β-acid-derived oxidation products remain probably undiscovered (130) reason why, taking into consideration the recent advances on compounds identification on the hop plant and their putative biological activity, research efforts should be directed not only in the continuity of the discovery of new compounds but also on the evaluation of their bioactivity, bioavailability and mechanisms of action of the molecules. Moreover, the optimization of analytical methods targeting these molecules should also be pursued as essential tools in evaluation of the amounts present in hops and beers as well as to assist in bioavailability assays.

Besides bitter acids-derived oxidation products other hop metabolites were recently identified. In the cones of the variety CAS, cultivated in northern Italy, researchers discovered very recently two new prenylated phloroglucinols (4-hydroxycolupulone and cascadone) and humudifucol (Figure 1.10), the first example of prenylated dimeric phlorotannin found in nature. These compounds were evaluated for their potential bioactivities on two enzymes related to inflammation (prostaglandin E_2 synthase and 5-lipoxygenase) involved in inflammation, pain, atherosclerosis and tumorigenesis. In resume, 4-hydroxycolupulone presented the highest inhibitory activity over the referred targets being comparable to the well-known anti-inflammatory action of xanthohumol itself. These findings concerning the newly discovered molecules confirms that hops should be regarded as a further source of molecules associated with anti-inflammatory and cancer chemopreventive activities.

Figure 1.10: Molecular structure of the newly discovered molecules 4-hydroxycolupulone, humudifucol and cascadone.

1.5.3. Research on new varieties

The number of released varieties has increased exponentially in the last years. New hop cultivars are being released with a strong incidence in the United States however the same trend is verified in other locations worldwide with new hop cultivars coming from Germany, United Kingdom, Australia, New Zealand, France, Czech Republic, Slovenia and Ukraine. Although this proliferation of varieties is beneficial to the beer overall evolution, since they are quality and differentiated products. The increasing number of hops will create a challenge to brewers, breeders, growers and suppliers, namely in what concerns the supply security, once less acreage can be allocated to each variety, and also because the demanding for a particular variety can change in a few years, making difficult to the growers define their planting plans.

Notwithstanding the search for new varieties with more pest and disease resistance, as well as strong commercial qualities, mainly at the aroma profile, bitterness, yield and storage attributes, will pursue due to the vitality of the current beer environment. Most of the varieties presently used were directly developed from domestication of wild ancestor plants, whilst modern agricultural breeding techniques rely on precise crossing using well-known male and female plants to develop new varieties. In fact, hop research institutes and big hop producing companies are investing in the development and release of new hop varieties with valuable agronomic characteristics, as referred, but principally focused on organoleptic characteristic valued by modern brewers.

The modern development and release of a new variety involves a huge amount of work, as well as, financial resources and specific facilities, and takes not less than 11 years to

finalize. It starts with the parental selection, i.e. choosing the males and females that will be used as germplasm sources. Those are selected based on the characteristics required for the new plants and considering that some will be present in the new plants. However, due to the uncertainty about which selected features will be expressed in the new plants, about 40,000 genotypes are usually tested during the selection process (https://ychhops.com). After the parental choice, seeds are germinated and the following 7-8 years are dedicated to early, intermediate and advanced selection throughout the use of tools that will permit the narrowing of plants down to one or two varieties that best represent the original breeding objectives. The last two years of the selection process are then spent in field trials, expanded to more cultivated area. The last step compromises the test of some hops in experimental brewing before the final release of the variety.

The large efforts to develop new varieties imply the necessity of resorting to commercial protection strategies, that will be reflected on the hops price and make that variety more valuable, hence a target to unscrupulous dealers who can create an authenticity issue in hops trade. A big concern of growers and brewers is the varietal authenticity of the plants used. Therefore, some hops identification has been increasingly applied, being the molecular DNA-based techniques claimed as methods of choice to identify or differentiate wild hops found in the nature, commercialized varieties, or new varieties released (4, 27). These techniques are developed at some hop research labs and institutes worldwide and can be used to plant certification. DNA analysis has the advantage of permitting the identification of the variety from any part of the plant that contains DNA, both growing in the field and in dried and processed forms, and can detect as little as 5% of another variety (57).

The chemical analyses of the essential oil and of resin components evaluates hops quality, and can give valuable information to sustain the botanical identification of a hop varieties. However, varietal identification only by analyses of the hops chemical composition usually involves detailed statistical treatment by chemometric analyses, in order to distinguish from similar varieties (133), and do not provide an unequivocal identification of the varietal. Since, phenotypical changes of chemical markers are strongly influenced by environmental conditions during plant growth (region, soil, harvest time, agricultural practices, environmental factors), cones processing and storage. Thus DNA-based methods were developed targeting the plant phylogenetic studies and hops molecular identification, to circumvent the environmental variation of chemical markers. Several methods were proposed for hop varietal authenticity control as the AFLP - Amplified Fragment Length Polymorphism (134); RAPD - Random Amplified Polymorphic DNA (135), SSR – Simple Sequence Repeats (9, 136-139) and DArT - diversity array technology markers (140). Although several methods are available the analysis of Single Nucleotide

Polymorphisms (SNPs), differences of single nucleotide in some homologous DNA among varieties, is presently considered the more reproducible tool for the identification of varieties of several plants (141). Next Generation Sequencing (NGS) technologies applied to the study of the hop genome showed the presence, in average, of a SNP in every 346 bp of the hop genome (142). These authors identified 17128 SNPs in the hop genome and concluded that a group of 3068 SNP retrieved the same level of discrimination amongst varieties. However, the minimum number of markers to differentiate among all genotypes was not identified. Notwithstanding, Henning and collaborators (2015) identified 7 SNPs from a group of 374829 markers that effectively differentiated all 121 varieties and accessions (representing a broad spectrum of hop lines from around the world).

Therefore, these authors proposed the use of high-resolution melting analysis (HRMA) curve analyses as a simple, rapid and more economically viable means to perform genetic fingerprinting on hop genotypes (10).

In general, the present chapter revises the most relevant traded hop varieties, their chemical, biological and brewing characteristics, as well as the analytical methods used to assure hop quality and authenticity. The current international hop market was characterized, and trends of hops trade developments were approached.

It is irrefutable that the hop market is on the threshold of fundamental change, which is being driven by the internationally growing preference for differentiated beers, in which production more hops are used, this is mainly sustained by the craft brew sector. This movement has led to an increasingly search for new flavours in beer, therefore new hops that provide these flavours are required, motivating the search for the new “green gold”. Several new varieties are being developed and will be released in the next few years, which promotes the development and enlargement of studies about their behaviour in beer, considering also the new forms in which hops are used. Moreover, the increase in hops usage, either in quantity or on the number of differentiated varieties, will certainly increase the bioactivity of the beer itself, which by its hand will motivate more studies concerning the bioactivity of beer.

In Portugal, a great number of hop wild populations were identified and their morphologic characterization revealed large variability (11). However, no published studies were found concerning their genetic variability, volatile compounds profile and sensory characteristics. Hence, this thesis was conducted to provide new insights about chemical, biological and sensorial characterization of hops and dry-hopped beers. Searching for new perspectives for the use of native Portuguese hops and evaluate their brewing quality. In this context, Portuguese native and commercial hops were compared by DNA analyses, using a minimal model with 7 SNPs to genotype the population. Samples were also discriminated by near- and mid-infrared vibrational spectroscopy, which are regarded as

rapid, cost-effective, non-destructive potential methods to be used as an authenticity tool of hop varieties. Moreover, main differences were pointed by analyses of volatile profile and aroma attributes. Even more, emphasis was given to dry-hopping techniques to optimize and predict the extraction of volatiles and bioactive compounds (α-acids and xanthohumol). Therefore, beers were dry-hopped with selected Portuguese genotypes and some commercial varieties. Sensory impression, volatile composition, as well, quantification of α-acids and xanthohumol, on hops and dry-hopped beers, were evaluated. Furthermore, was assessed the antiproliferative activity of xanthohumol, isoxanthohumol, α and β-acids, and iso-α-acids, both as pure compounds and as part of the beer matrix on colon Caco-2 cells.

PART II

Hops discrimination/ characterization

CHAPTER 2

Portuguese wild hops genotyping by high resolution melting analysis of a minimal SNP set

2.1. Introduction

The hop plant (*Humulus lupulus* L.), together with other species from the genus Humulus, is probably originated from China, from where it migrated to moderate climatic zones of the northern hemisphere being indigenous to Europe, Asia and North America, spread in humid and low altitude areas (39). Wild hops are classified accordingly to leaf morphology into five taxonomic varieties; var. *lupulus* for European wild plants and cultivars, var. *cordifolius* for Japanese wild hops and var. *neomexicanus*, *pubescens* and *lupuloides* for North American hops (28).

The female inflorescence of hops, the hop cone, is the ingredient that adds bitterness, microbial protection, foam stability and substantially contributes to the flavour of beer (1) being this last one of the most important attributes in the definition of premium hop varieties and beers. Therefore, breweries around the world are constantly in search of different hop varieties to produce beers with differentiated flavour profiles. Motivated by this trend, an exponential growing has been observed in the breeding of new varieties following classical agronomical techniques as crossing well-known genotypes with new sources of genetic variation. Wild germplasm is many times used a source of such variability used in cross-breeding; notwithstanding, wild plants can also be directly domesticated, when they spontaneously present adequate agronomic characteristics (e.g. productivity and disease resistance) as well as the bitterness or aroma required for beer production (4, 5, 143). Wild hops have the additional advantage of being resistant or tolerant to endemic pathogens (27). In fact, hop breeding over the last years has been performed by hybridization of different germplasm (including varieties) or by clonal selection of wild hops from different regions (133). The above referred configures the main the reason for the existing interest of researchers and brewers in the prospection of wild genotypes.

Wild populations of hops are, thus, being characterized all over the world at the chemical, organoleptic (aroma profiles) and molecular levels, providing the basis of genetic selection for breeding. Hops can be generally considered extremely polymorphic species, which is justified by the plant easiness of cultivation, rustic habits and high degree of intraspecific genetic variability induced by the adaptation of the plant to different climatic and ecological conditions (4, 28, 143, 144). Also, breeders have improved some varieties by phenotypic selection, however, since phenotype depends not only on the genotype, but also on the environment, the phenotypic selections is as imperfect measure of the genetic potential (145). The genetic characterization of germplasm is, thus, necessary and in many cases is the first step for its proper evaluation. It configures a way to unequivocally assess the diversity which, when high, and clearly differentiated from the classical varieties, can be suggestive of richness and variability at the organoleptic and chemical levels. Genetically

diverse germplasm is in general taken as a good clue for its commercial potential in beer production.

Genetic characterization of hops is being made in the last years using multiple markers similarly to other agricultural crops. The first works reporting genetic analysis of hops (135) applied the Random amplified polymorphic DNA (RAPD) technique, by the time frequently used, and tested it in seven varieties (Aquila, Banner, CAS, CLU, FUG, MTH and WIL). Authors found the procedure most valuable for testing planting stock before establishing a new hop yard. Since then several other works were performed in hops characterization using RAPDs including the evaluation of the genetic variability and relationships of 54 hop cultivars from all the major hop growing regions in the world (146) who described the division of cultivars into American and European groups. Other authors opted by using several methods as RAPD, STS - Sequence Tagged Sites, ISSR - Inter-Simple Sequence Repeat and AFLP - Amplified Fragment Length Polymorphism to test its potential in the use of the genetic characterization of 10 hop varieties (144). Pillay and collaborators (1996) made use of the Restriction Fragment Length Polymorphism (RFLP) technique, applied to ribosomal RNA genes, in the characterization of 118 cultivated and native European, Chinese and North American genotypes. European and Asian genotypes presented predominantly one genetic type, North-American cultivars were related to a second group and North-Americans natives were included in a third group. Another research group (28) studied the molecular phylogeny of hops by sequencing ribosomal DNA intergenic spacers and several chloroplast DNA non-coding regions in which work authors found considerable genetic diversity between wild hops form each growing region.

Although several markers have been used in hops, the most common ones are the microsatellites or SSR (Simple Sequence Repeats). In a work evaluating 182 hop accessions (wild European and American; and cultivars), 7 nuclear and 32 chloroplast SSR markers were screened (147). Researchers concluded that genotypes were separated in two big groups one including European wild accessions and cultivars and a second group American wild accessions. Also in 2010, Patzak and collaborators (143) genotyped at 9 SSR a total of 217 wild hops including plants form Canada, US , Europe and Caucasus regions. As in previous publications wild hop genotypes were divided into two major groups, North American and Eurasian this last sub-divided into two groups corresponding to continental Europe and the Caucasus region. In another work these authors found no correlation of chemical characteristics with genetic diversity assessed by SSR markers (4). A few years later, another team increased the number of available SSR markers by identifying 952 new *loci* and successfully tested them in 8 cultivars (139). SSR were also used by Italian researchers (133) to characterize 22 wild hop accessions collected from distinct populations. The selected germplasm was probed with 9 SSR *loci* and compared with 5 commercial

varieties (CBS, FUG, HMG, TET and WIL) revealing a genetically heterogeneous pool. Spanish hops (75 Galician wild genotypes) were also subjected to SSR genotyping at 7 loci and compared with wild Europeans, wild Americans and cultivars. Authors identified a genetic structure in 2 groups, one composed of the wild American genotypes and the other including all the others, as reported by several other works. Italian wild hops genotypes were also evaluated in a recent work (148) using 9 nuclear SSR markers in 80 wild samples and 43 European and US cultivars, which were separated in two groups, one including most of the European and US cultivars and another the Italian wild hops. Besides its use for genotype diversity characterization some DNA-based methods, including SSR markers, are used to distinguish males from females, which is also of great importance for breeding, particularly when evaluating germplasm in earlier phenological state (145, 149).

More recently, researchers initiated the use of Single Nucleotide Polymorphism (SNP) markers in hop genetics analysis. A single nucleotide polymorphism (SNP) is a nucleotide position, which is variable across the genome, resulting in mutations that produce base-pair differences among chromosome sequences. Their high abundance and genome-wide distribution make them a valuable source of genetic variation for population demography, adaptation, and evolution (150). Moreover, as they are not limited to clustered region as SSR are therefore more representative of the genome (151). Scarce works were published with interesting results concerning SNP evaluation in hops, as the one of Matthews and collaborators, which identified and validated 17128 next generation sequencing derived SNPs in the hops genome with high potential in assisting hop breeding at several levels (142). A reduced group of 3068 SNP was identified, which resulted in a dendrogram similar to that obtained with the markers totality, but the definition of a minimal number of markers required to differentiate all genotypes analysed was not achieved. This was only accomplished by Henning and colleagues (10) that defined a very reduced set of markers (7 from an initial set of 374829 SNP previously filtered to 1006 found in all cultivars).. These 7 markers could differentiate all the 116 varieties and accessions included in the study.

The native populations of *H. lupulus* were already genotyped in several countries as Canada, Czeck Republic, Italy, Japan, Spain, and US, as referred above. In Portugal, a great number of hop populations were identified about twenty years ago, and a representative collection was installed at Portuguese Vegetal Germplasm Bank (BPGV) being maintained, properly treated for viral infections, and vegetativelly propagated since then. The collection was morphologically characterized and revealed large variability (11). However, no published studies were found concerning the molecular genetic characterization of the collection. The aim of the present work was thus to genotype the Portuguese population of hops and compare the genotypes with well-known commercial varieties. The minimal set of SNP markers proposed by Henning and collaborators were chosen for that purpose based

on the bibliographic revision mentioned above and on the availability of equipment in the laboratory as well as the possibility to high throughput analysis allowed by the technique.

2.2. Materials and methods

2.2.1. Vegetal material

A total of 143 genotypes were included in the present study (Supplementary Table 1). Young leaves of 90 accessions of *Humulus lupulus* L. (coded as PTG), harvest 2015, were collected at the Banco Português de Germoplasma Vegetal (BPGV, Braga, Portugal); 20 wild populations were gathered from the natural environment being some of them collected in the exact same locations of the original collection (codded as PTW); and 33 varieties originated from different countries (International Hop Growers Convention codes) were provided by the Slovenian Institute of Hop Research and Brewing (Žalec, Slovenia). Leaves were dried and stored until the moment of analysis according to the described in Appendix I.

The hop collection at the BPGV was established after systematic surveys and collecting missions, carried out from the North to the South of the country, during the period of 1997 to 2000. Spontaneous hop has a generalized distribution in the Northern and Centre parts of the country, however it was not found in the regions bellow the Tagus River (11). Table 2.1 presents the list of the wild Portuguese genotypes codes as well as the district of collection points.

Table 2.1. List of the wild Portuguese genotypes codes as well as the district of collection points. Na: "Not Applied": no information was provided in original records. *Collected in the same population/location of the original campaign.

Code	*District*	Code	*District*	Code	*District*
PTG01	*na*	PTG39	*Guarda*	PTG81	*Braga*
PTG02	*Braga*	PTG40	*Viseu*	PTG82	*Viana do Castelo*
PTG03	*na*	PTG41	*Viseu*	PTG83	*Viana do Castelo*
PTG04	*na*	PTG42	*Viseu*	PTG84	*Aveiro*
PTG05	*Viana do Castelo*	PTG44	*Braga*	PTG85	*Coimbra*
PTG06	*Coimbra*	PTG45	*Braga*	PTG86	*Guarda*
PTG07	*Coimbra*	PTG46	*Braga*	PTG87	*Porto*
PTG08	*na*	PTG47	*Viana do Castelo*	PTG88	*Porto*
PTG09	*Coimbra*	PTG49	*Vila Real*	PTG89	*Braga*
PTG10	*Coimbra*	PTG50	*Porto*	PTG90	*Braga*
PTG11	*Coimbra*	PTG51	*Vila Real*	PTG91	*Viseu*
PTG13	*Bragança*	PTG52	*Bragança*	PTG92	*Viseu*
PTG14	*Bragança*	PTG53	*Aveiro*	PTG93	*Leiria*
PTG15	*Guarda*	PTG54	*Viseu*	PTG94	*Leiria*
PTG16	*Guarda*	PTG56	*Viseu*	PTG95	*Lisboa*
PTG17	*Guarda*	PTG57	*Viseu*	PTG97	*Santarém*
PTG18	*Bragança*	PTG58	*Coimbra*	PTW01*	*Braga*
PTG19	*Braga*	PTG59	*Coimbra*	PTW02*	*Porto*
PTG20	*Porto*	PTG60	*Coimbra*	PTW03*	*Castelo Branco*
PTG21	*Porto*	PTG61	*Viseu*	PTW05	*Angra do Heroísmo*
PTG22	*Porto*	PTG62	*Santarém*	PTW06	*Angra do Heroísmo*
PTG23	*Viana do Castelo*	PTG63	*Santarém*	PTW07	*Bragança*
PTG24	*Braga*	PTG64	*Santarém*	PTW08	*Porto*
PTG25	*Braga*	PTG65	*Santarém*	PTW09	*Porto*
PTG26	*Bragança*	PTG66	*Santarém*	PTW10	*Vila Real*
PTG27	*Viseu*	PTG67	*na*	PTW11	*Bragança*
PTG28	*Porto*	PTG68	*Porto*	PTW12	*Porto*
PTG29	*Braga*	PTG69	*Bragança*	PTW13	*Porto*
PTG30	*Coimbra*	PTG71	*Viana do Castelo*	PTW14	*Castelo Branco*
PTG31	*Coimbra*	PTG72	*Braga*	PTW15	*Porto*
PTG32	*Aveiro*	PTG73	*Braga*	PTW16*	*Bragança*
PTG33	*Castelo Branco*	PTG74	*Braga*	PTW17	*Braga*
PTG34	*Castelo Branco*	PTG75	*Guarda*	PTW18*	*Viseu*
PTG35	*Guarda*	PTG76	*Viseu*	PTW19	*Leiria*
PTG36	*Guarda*	PTG77	*Vila Real*	PTW20	*Leiria*
PTG37	*Guarda*	PTG79	*Viseu*	PTW22	*Porto*
PTG38	*Guarda*	PTG80	*Vila Real*		

Figure 2.1 shows the geographical points were the BPGV hop collection was gathered. Not all the genotypes at the collection are indicated since for some of them it was not possible to obtain the geographical coordinates of the collection point.

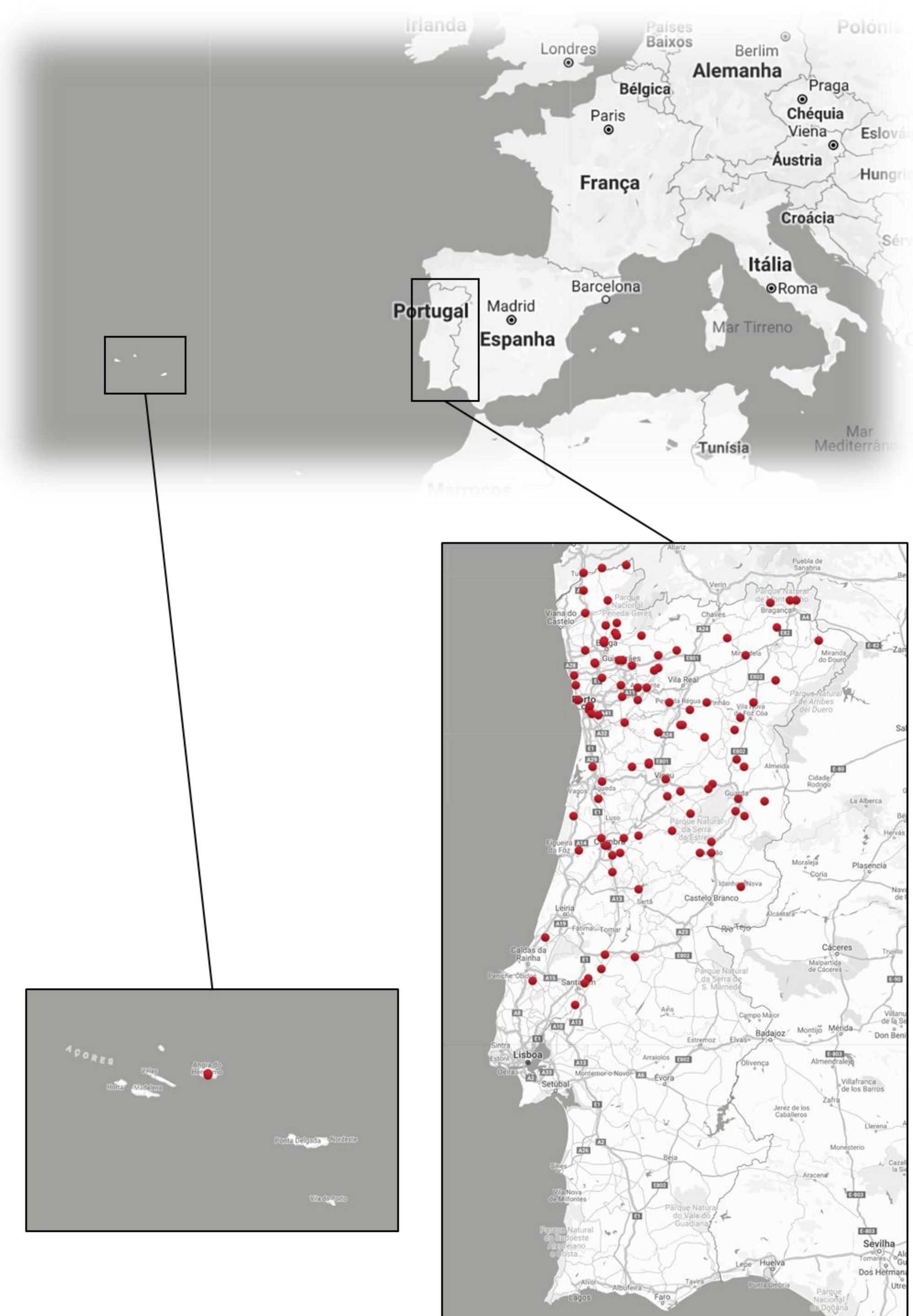


Figure 2.1. Collection places of the original germplasm maintained at BPGV (Portugal mainland) and an additional genotype collected in Azores islands. Red dots correspond to both the samples collected in BPGV (PTG) and directly in the original locations (PTW). Geographical data was retrieved from the work of Rocha (11).

2.2.2. DNA extraction and quantification

DNA was extracted from 50 mg desiccated leaves using the commercial kit for plant DNA extraction (E.Z.N.A.® HP Plant DNA Mini Kit, Omega Bio-Tek, Norcross, GA, US). Samples

were homogenised together with the corresponding extraction buffer in a Precellys Evolution laboratory mill at 7500 rpm (Bertin instruments, France) using 2 ml screw-capped tubes containing ten 1.4 mm ceramic beads. Extracts were quantified and evaluated for purity by UV absorption at 260 and 280 nm in a 4 µL sample aliquot using a BioTek Synergy HT plate reader (BioTek, US) equipped with the Take3 Micro-Volume Plate adapter from the same supplier. DNA integrity was verified in 0.8 % agarose gels stained with GelRed® (Biotium, Fremont, CA, US).

2.2.3. Real-time amplification and melting analysis

Real-time amplification reactions were conducted in a Bio-Rad CFX 96 thermalcycler using the SsoFast™ EvaGreen® Supermix (Bio-Rad, CA, US), in 10 µL volumes in white 0.2 ml 8-tube strips or 96 well plates (Bio-Rad, CA, US). PCR conditions were optimized to reach the shortest time of analysis, defined as: an initial denaturation of 98 °C for 2 min, followed by 35 cycles of 98 °C for 5 s and the annealing temperature (from 57 to 63 °C) for 5 s. Immediately after PCR, melting analysis was performed from 60 to 95 °C in 0.5 °C increments. Melting curve clustering was performed using the Precision Melt Analysis™ v 1.0 Software from Bio- Rad (Hercules, CA, US). Primers for the amplification of the 7 markers TP137094, TP15403, TP245055, TP295074, TP400349, TP411590 and TP437202 were the ones published by Henning and collaborators (2015) except for the SNP TP245055 and TP411590 to which alternative primers were considered, due to a weak amplification obtained with the published ones. New primers were designed using the Primer3web online tool (http://primer3.wi.mit.edu/) with the following sequences: TP245055alt_forward: TGCCTCCTTTGACCGTTAAG; TP245055alt_reverse: ATCTCAGTGGGGTTCCTTCC; TP411590alt_forward: CCCTGAATGACCCTCAATGT; and TP411590alt_reverse: AGGGTTCGTTTGGTTATTCC. These permitted the amplification of fragments of 82 and 90 bp, respectively.

2.2.4. SNP calling and phylogenetic analysis

The varietal genotyping was done at the 7 referred SNP markers proposed by Henning and collaborators (2015) as the minimal set required to differentiate all the commercial hop accessions tested in their study. SNP calling was done by assigning each melting curve to one of the three allelic combinations for each SNP site with a confidence of at least 98%. SNP data was concatenated in a fasta file and aligned using the program MEGA X (Kumar et al., 2018) also used to calculate the overall genetic variation expressed as mean pairwise distances. Phylogeny reconstruction was performed using the UPGMA (Unweighted Pair

Group Method with Arithmetic mean) method and the Kimura algorithm, using the online tool provided by European Bioinformatics Institute (EMBL-EBI) for simple phylogeny (https://www.ebi.ac.uk/Tools/phylogeny/simple_phylogeny/).

2.3. Results and discussion

DNA extracted from leaves was of high purity with an average absorbance ratio at 260/280 nm of 1.9 and a mean concentration of 408 ng/µL. These values permitted the dilution of samples to optimal DNA concentration of 10 ng/µL which is very favourable to PCR amplification since it enables a strong co-dilution of inhibitors.

In the present study the SNP allele discrimination was done using the High-Resolution Melting Analysis (HRMA) technique. HRMA provides a rapid, simple, high-throughput, cost-effective, and alternative single-tube approach to the direct DNA sequencing for the detection of SNP which are particularly useful if many samples are to be analysed. This novel technique has been successfully applied in many different research areas, as far as analysis of cancer-related mutations, genotyping of pathogens, and plant genotyping (152). In fact, the researchers that identified the set of 7 minimal markers for hop genotyping (10) chosen in this work proposed themselves the genotyping of hops using this technique. HRMA is performed in close tube following the PCR amplification requiring no further manipulation. The process is typically fast and can be completed in less than 1hour. It generates DNA melt curve profiles, which are both specific and sensitive enough to distinguish nucleic acid variation of known variants (targeted genotyping) as SNP (153). The different genotypes are most easily discerned by plotting the fluorescence difference between normalized melting curves. One melting curve is chosen as a reference, and the difference between each curve and the reference is plotted against temperature to forming a “difference” plot. The original reference curve became a horizontal line at zero, and different genotypes clustered along different paths for easy visual discrimination (154).

In the present work a robust amplification was obtained for all the markers and most of the samples which allowed a good separation of genotypes accordingly to the melting profile of the differences curves as can be observed in Figure 2.2 regarding the marker TP137094.

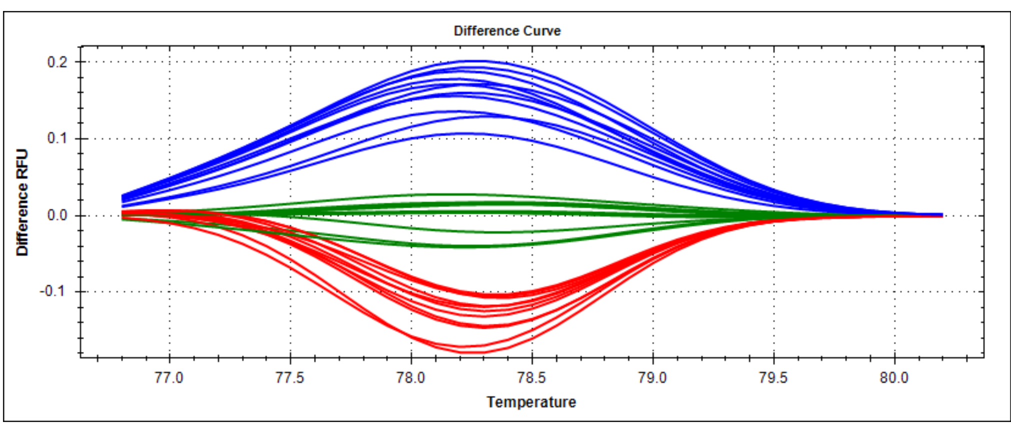


Figure 2.2. Example of a HRMA differences curve graph obtained for 30 different varieties for one *locus* (TP137094). The three displayed melting profiles correspond two each of the possibilities for allele combination for heterozygote and homozygote varieties: GG (blue), GA (green) and AA (red).

Each sample originates a melting profile, for each SNP, corresponding to a unique genotype. SNP data from all the samples and markers were concatenated in a fasta file and a simple UPGMA phylogenetic three was plotted as referred previously (Figure 2.3).

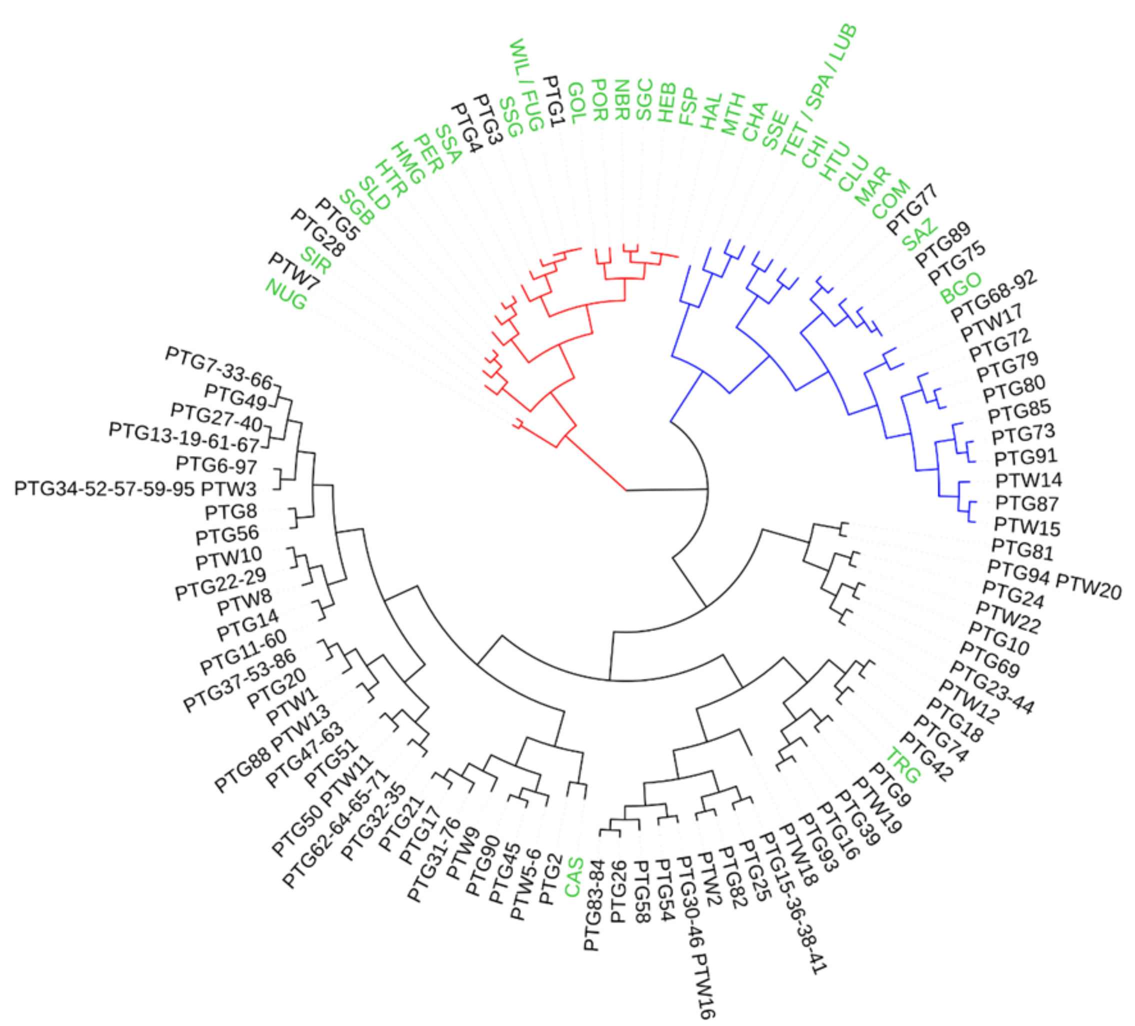


Figure 2.3. UPGMA dendrogram of the 143 analysed genotypes. Samples presenting the same genotype were grouped to allow a clear dendrogram presentation. Branch lengths are representative of the genetic distance. Genotypes in black characters are of Portuguese origin and in green are represented the commercial varieties using the IHGC codes.

From Figure 2.3 it can be inferred that most of the 143 genotypes analysed (ca. 75%) could be clearly differentiated demonstrating the discriminant power of the 7 markers. These were distributed in three main clusters specified by red, blue and black branch colours in the dendrogram. Data obtained herein in general reveals that the analysed pool was composed by genetically different and heterogeneous material.

The biggest cluster, in black, included 91 genotypes and is composed almost exclusively (ca. 98%) of Portuguese wild accessions apart from the commercial varieties CAS (US) and TRG (UK). This configures a clear differentiation of the wild genotypes and demonstrates the genetic uniqueness of the Portuguese germplasm. The presence of the variety CAS (US) within the wild hops group was also verified in the work of Rodolfi and collaborators (148) in a study of Italian wild genotypes. In another work the variety was similarly placed

separately along the dendrogram not clustering with any other variety (146). These facts suggest some differentiation of the CAS variety from other cultivars which can somehow be the reason for its inclusion in the Portuguese wild group.

The two other clusters, blue and red, have similar sizes including 28 and 24 individuals, respectively. The blue cluster is composed of approximately half native genotypes (54%) and the other half of varieties (46%) originated from several countries as UK, US, Germany, Poland, Czech Republic. Grouping of commercial varieties following their country of origin was not verified, as referred by several previous works (10, 147, 148) possibly because there is a major influence of the wild European genotypes among hop cultivars all over the world. These works, in which the relationships between American, European wild genotypes and varieties are studied, report a genetic structure consisted of only two groups: one with the American wild genotypes and another containing the European wild genotypes and the varieties. In fact, cultivars and wild European hops share the same genetic background since many of the cultivars mostly used nowadays have their origin on selection over local European plants (27). Moreover, some authors found a proportion of genetic variability in European wild hops lower than in American and Caucasia wilds which suggests a possible introgression into the natural populations during the historical long cultivation period in Europe (4).

Finally, the red cluster is composed mainly by commercial varieties (75%) and about 25% of Portuguese genotypes. Concerning these 6 Portuguese native hops, integrated in the cluster containing mostly varieties, 3 are from unknown locations, 2 from districts bordering abandoned hops plantations in the Northwest of Portugal, and particularly the PTW7, very closely related to NUG, was collected in a location nearby an active hop farm in the Northeast of the country. The presence of these wild hops within the varieties group may indicate the occurrence of cross-pollination of male wild genotypes and female commercial varieties, in local hop farms, resulting in new genotypes that, for some reason, were dispersed in farms surroundings.

In the work of Henning and colleagues (2015) the 7 SNP markers were able to discriminate all the 116 analysed varieties. This full discrimination was not verified in the present work since our results could not achieve the separation of the groups of samplesTET, SPA and LUB and of WIL and FUG (Figure 2.3). In the first case we cannot fully compare the results with the work of Henning since the variety SPA was not included in that study. Concerning TET and LUB they were discriminated previously but are located in the same cluster, thus closely related, which lead us to the conclusion that an intravarietal mutation in one of the 7 SNP or even an error in the HRMA SNP calling could occur (153). In what respects the group FUG and WIL their similarity for the present 7 markers is highly probable to occur since WIL is an offspring of FUG (133). Moreover, Henning studied

Fuggle N and Fuggle H, which are a clonal selection and a Hybrid from FUG, respectively, not the original genotype (selected form European wild hops) included in our study. Besides that, all varieties could be discriminated with a similar distribution in the dendrogram clades as is the case of eg. PER and the German genotypes HTR and HMG, clustering in the same group in both works.

Another point that should be highlighted is the genetic relatedness of the 5 wild genotypes maintained at the BPGV and the ones that were collected in the same geographical locations (Table 2.1, genotypes indicated with *). These were collected from the same populations however 20 years latter (2017) and in only one case, the PTW3, which corresponds to the PTG34, both genotypes were equal. All the other pairs were different (PTW1/PTG19; PTW2/PTG22; PTW16/PTG69; PTW18/PTG56) although clustering in the same big cluster of the wild populations. This fact indicates that hop plants are cross-pollinating in the wild with different populations in most of the cases, as expected, reason why the preservation of the Portuguese germplasm collection under controlled conditions with vegetative propagation is of top importance. In addition, the collection was perfomed in the same local but very unlikely in the same plant.

Concerning the genetic variation, generally expressed as mean pairwise distances were of 0.34 (base differences/site) for the entire set, 0.29 within the Portuguese hops and 0.36 in commercial varieties. Results confirmed the high intraspecific variability of the Portuguese germplasm as well as the efficiency of the 7 proposed markers in the intraspecific discrimination of hops. The somewhat lower genetic variation within the Portuguese wild hops is in accordance with previous publications, concerning other European wild genotypes, since more variation is found in Americans wild genotypes (meanwhile transferred to the genetic pool of current varieties by breeding) than in European hops (4, 10, 27, 143, 145). The hypothesis of the current hop genotypes being originated from a single genetic bottleneck event is under discussion (145).

2.4. Conclusions

To sum up, results described here configures the first genetic characterization, at the DNA level, of the biodiversity of the Portuguese collection of native hops; and the application of a minimal set of 7 SNP markers recently proposed for the characterization of hop germplasm using the high throughput, fast and economically attractive HRMA method. Cluster analysis of 110 wild hop genotypes and 33 commercial cultivars revealed that the pool was composed by genetically different and heterogeneous material that should be further characterized at the phytochemical level throughout chemical analysis of the main

parameters of interest to brewers, including the highly important volatile profiles. Most of the 143 genotypes analysed were discriminated evidencing the power of the minimal set of SNP however, future research should require the use of much more markers as the application of Next Generation Sequencing to SNP genotyping. The clear discrimination of a big group composed almost exclusively by Portuguese wild genotypes suggests some specificity of the genetic pool that should be further investigated; for instance, by adding data from other European wild genotypes as well Asiatic and American wild specimens. Results presented here are relevant and contribute to the affirmation of the high richness potential of Portuguese native hops as starting breeding material to initiate a national genetic improvement program.

CHAPTER 3

Hops discrimination by NIR or MIR infrared spectroscopy: commercial versus Portuguese native hops

3.1. Introduction

The identification of hop varieties can be performed by different strategies, such as, (i) molecular analysis (4), which is expensive, (ii) evaluation of morphological characteristics (133), difficult to achieve when hop cones are processed, and by (iii) chemical analysis of hop compounds, namely bitter acids, flavonoids, essential oils, or proteins, individually or using the combination of different chemical groups (155-163).

In general, the techniques used for hop identification require a considerable amount of sample and are destructive. To avoid those drawbacks, vibrational spectroscopic techniques, namely near infrared (NIR) and mid-infrared (MIR) spectroscopy, emerge as suitable alternatives for hops discrimination. The NIR spectroscopy results from overtones and combination of fundamental vibrational bands, namely C-H, N-H, O-H and S-H bonds, in the spectral range of 14,000 to 4,000 cm^{-1}. MIR spectroscopy is characterized for fundamental, bending and rotating vibrations in the spectral range of 4,000 to 400 cm^{-1}. The MIR spectrum has a higher specificity than NIR spectrum and for this reason is considered more appropriate for identification and characterization purposes. Both these techniques are rapid, non-destructive with a low-cost per analysis (164). There are already some examples in the literature exploring vibrational spectroscopic techniques for discrimination purposes, namely in genetically and non-genetically modified maize plants (165), grapevine varieties (166), bacteria species discrimination (167), and even hops (162) although focusing only a small sample size usually within the same country of origin.

The aim of this study was thus to evaluate if NIR and MIR spectroscopy can be used as fast and non-destructive technique for the differentiation and identification of hop varieties targeting authenticity purposes. A total of 33 commercial varieties (representing about 75% of the total volume commercialized worldwide) and 76 Portuguese native hops were analysed. DNA-based SNP characterization was performed as a way to warrant the differentiation of commercial varieties.

3.2. Materials and methods

3.2.1. Chemical and materials

International Calibration Extract (ICE-3), containing a mixture with 44.64% of α-acids (humulone, cohumulone and adhumulone) and 24.28% of β-acids (lupulone, colupulone and adlupulone) was acquired from Labor Veritas (Zurich, Switzerland). Xanthohumol standard ≥ 99% purity was purchased from Extrasynthese (Z.I Lyon Nord, France). Formic

acid (98–100%) and sodium acetate was supplied by Merck (Darmstadt, Germany). Methanol and Acetonitrile were HPLC grade (≥ 99,9%). Water was purified with a Milli-Q System (resistivity >18 MΩ cm) (Millipore, Bedford, MA, US).

3.2.2. Hop samples

A total of 165 commercial samples (five for each 33 hop varieties) were purchased from Sovina (Porto, Portugal) brew store and coded as described in Appendix I. Table 3.1 presents the content of α-acids, as indicated by the manufacturer. The choice of these varieties was done by selecting the most relevant in terms of market share accounting for 75% of the worldwide market volume in 2016 (168). Moreover, 76 Portuguese native hops were collected and coded as described in Appendix I. This study included the harvest years of 2015 and 2016 for commercial and 2016 for Portuguese hops.

Table 3.1. Variety designation, α-acids content (%) andorigin of the 33 commercial samples studied.

Commercial variety	α-acids (%)
Amarillo® (VG1)	9
Bobek (SGB)	5
Bramling Cross (BRX)	4
Brewers Gold (BGO)	4.7
Cascade (CAS)	6.9
Challenger (CHA)	6.5
Chinook (CHI)	12.5
Citra® (CIT)	13
Cluster (CLU)	7.5
Crystal (CRY)	6
East Kent Golding (EKG)	5.2
Ella (ELL)	14
Ekuanot® (EKU)	13.4
Fuggle (FUG)	5.2
Goldings (GOL)	5.1
Hallertauer Magnum (HMG)	10.5
Hallertauer Mittelfrüher (HAL)	3.6

Commercial variety	α-acids (%)
Hallertauer Tradition (HTR)	5
Hersbrucker (HEB)	2.3
Mosaic® (MOS)	12.8
Mount Hood (MTH)	6.3
Nelson Sauvin™ (NSN)	11.9
Northern Brewer (NBR)	6
Nugget (NUG)	11
Perle (PER)	5
Saaz (SAZ)	3
Simcoe® (SIM)	13.1
Spalter Select (SSE)	5.2
Summit® (SUM)	15
Target (TRG)	8
Tettnanger (TET)	2.1
Tomahawk® (TOM)	16.5
Willamette (WIL)	4.7

3.2.3. MIR spectral acquisition

A Fourier transform infrared PerkinElmer Spectrum BX FTIR System Spectrophotometer (Waltham, US) equipped with a deuterated triglycine sulphate DTGS detector and an attenuated total reflectance (ATR) accessory, PIKE Technologies GladiATR™ (Madison, US), was used to collect the hop MIR spectra in diffuse reflectance mode. The spectra were acquired in the spectral range of 4,000 to 400 cm^{-1}, with a

resolution of 4 cm^{-1} and 32 scan co-additions. The hop samples were transferred to the ATR crystal and compressed until constant signal. For each hop cultivar, one small portion was transferred and a spectrum was collected for each portion. Therefore, a total of 5 spectra were acquired for each hop variety. Between each hop variety, the ATR crystal was cleaned and a background was acquired.

3.2.4. NIR spectral acquisition

A Fourier transform near infrared spectrometer, FTLA 2000 from ABB (Québec, Canada) equipped with an indium-gallium-arsenide (InGaAs) detector was used to collect the hop NIR spectra in diffuse reflectance mode. Each spectrum was recorded as the average of 64 scans in the spectral range of 10,000 cm^{-1} and 4000 cm^{-1}, with 8 cm^{-1} resolution. The hop samples were transferred into borosilicate flasks in order to perform spectra acquisition. The background was measured at the beginning of each analysis using a reference substance (Teflon). A total of 5 spectra were acquired for each hop variety.

3.2.5. NIR and MIR data analysis

The NIR and MIR data analysis was performed using principal component analysis (PCA) (169) and partial least squares discriminant analysis (PLS-DA) (170). PCA was used to detect outliers and find common patterns while the later was used to develop discrimination models. For PLS-DA models, samples were classified according to the respective hop genotype. The available data were divided in calibration (70%) and validation (30%) sets in a random way, but maintaining the same proportion of each variety in both sets (to avoid unbalanced hops varietyy classes in both sets) (171). The assessment of the optimal number of latent variables (LV) was done using the leave-one-sample-out cross-validation procedure using only the calibration set. The NIR and MIR spectra were divided in five spectral regions. The NIR regions were: R1 from 10,000 to 7,472 cm^{-1}, R2 from 7,468 to 6,083 cm^{-1}, R3 from 6,079 to 5,389 cm^{-1}, R4 from 5,385 to 4,964 cm^{-1} and R5 from 4,961 to 4,035 cm^{-1}. The MIR regions were: R1 from 4,000 to 2,782 cm^{-1}, R2 from 2,780 to 1,882 cm^{-1}, R3 from 1,880 to 1,492 cm^{-1}, R4 from 1,490 to 874 cm^{-1} and 872 to 600 cm^{-1}. These regions were tested individually and in combination for the selection of the best spectral regions. The selection of best processing technique involved testing standard normal variate (SNV) and Savitzky-Golay filter (with first and second derivatives, different filter widths and polynomial orders) individually and in combination. After the optimization

of the best spectral regions and pre-processing methods, the test set was projected in the optimized PLS-DA calibration model to assess the percentage of correct predictions. Model predictions were expressed as confusion matrices (171). The total percentage of correct predictions was obtained adding the diagonal elements of the confusion matrices. One PLS-DA model was developed for each spectroscopic technique (NIR and MIR). Loadings of the first four LV of each developed PLS-DA were also analysed to understand which specific wavenumbers were more important. Spectral data were mean centred before application of PCA and PLS-DA.

All chemometric analyses were performed with Matlab version 8.6 (MathWorks, Natick, US) and PLS Toolbox version 8.2.1 (Eigenvector Research Inc., Wenatchee, US).

3.2.6. DNA Analysis

DNA extraction, PCR amplification and HRMA of the SNP markers were performed as described in sections 2.2.2 to 2.2.4

3.2.7. Analyses of α-acids, β-acids and xanthohumol by reverse-phase liquid chromatography with ultra-violet diode array detection (RP-HPLC-UV-DAD)

To quantify the percentage of α-acids, β-acids and xanthohumol in Portuguese hops, extracts were prepared by maceration of 0.5 g of sample with 10 mL of methanol acidified with 1% formic acid (v/ v) for 30 minutes at room temperature, under stirring (30). Reverse-phase liquid chromatography was performed using an HPLC system from Gilson (France), consisting of two pumps (305 and 306), an 805 manometric module, an 811C dynamic mixer, an injection port with a 20 µL loop (Rheodyne, US) and a photodiode array detector (Prostar 335 DAD from Varian, US) controlled by a data processor software (Varian Stars Workstation, US). It was also used a YMC-triart C18 column (250x4.6mm, 3µm), with a mobile phase composed of (A) 5mM formic acid / Sodium format buffer pH 3.6 and (B) 2% of A solution in acetonitrile. The gradient elution started with 35% of B solution, rising to 75% of B from 3 to 23 min, and 75% to 100% from 23 to 40 min, then maintained for 5 min. Afterwards the percentage of solvent B was reduced back to 35% and re-equilibration was allowed over 10 min. The analyses were performed with a flow of 0.8 mL/min and 35 °C of column temperature. DAD data acquisition was made in a range from 220 to 600 nm.

3.3. Results and discussion

3.3.1. Validation of commercial samples by SNP discrimination

The varietal genotyping was assessed using 7 SNP markers (TP137094, TP15403, TP245055, TP295074, TP400349, TP411590 and TP437202) required to differentiate commercial hop accessions, as proposed by the study of Henning and collaborators (2015) (10). Amplicons of each marker originate three different HRMA curves corresponding each one to one of the allelic variants (two homozygotes and one heterozygote). SNP calling was performed by assigning each sample melting curve to one of the three allelic combinations for each SNP site. It was possible to achieve the complete discrimination of all the 33 commercial varieties in study and to confirm its phylogenetic relatedness (Figure 3.1), assuring this way that samples acquired are genetically different eliminating putative problems of cross mislabelling.

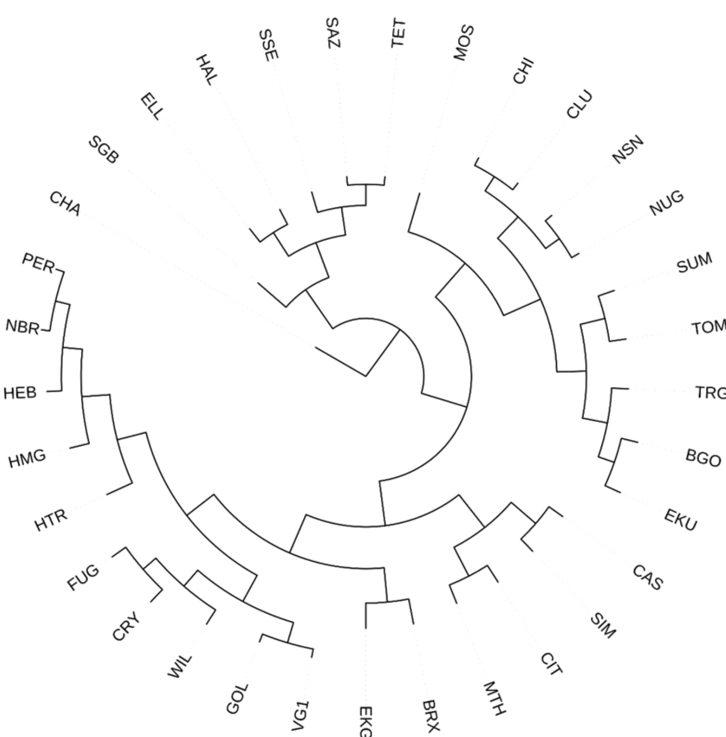


Figure 3.1. UPGMA dendrogram of the 33 analysed genotypes from commercial varieties.

3.3.2. Exploratory spectral analysis

The NIR and MIR spectra of commercial hop samples were depicted in Figure 3.2. As abovementioned, a PCA was performed with both NIR and MIR spectral data. The data was just mean-centred before application of PCA and the entire spectral regions were used. The results suggested that no outliers were identified in both data sets. Then, with the objective of understanding if the NIR and MIR spectra gather specific information regarding hop variety, the first two principal components scores were plotted (Figure 3.3). In the plots of both NIR and MIR scores it was visible some cluster formation tendency regarding hop's variety, suggesting that both NIR and MIR spectra contain information related with hop variety. The differentiation was not clear.

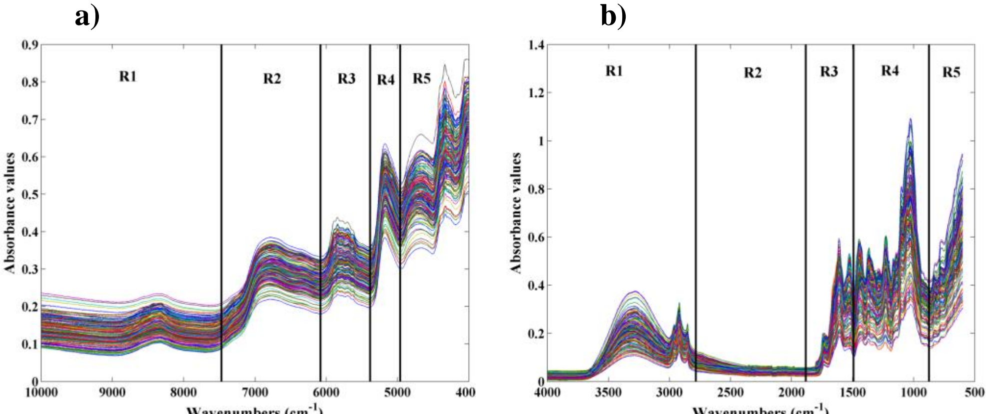


Figure 3.2. Hops raw spectra: NIR (a) and MIR (b).

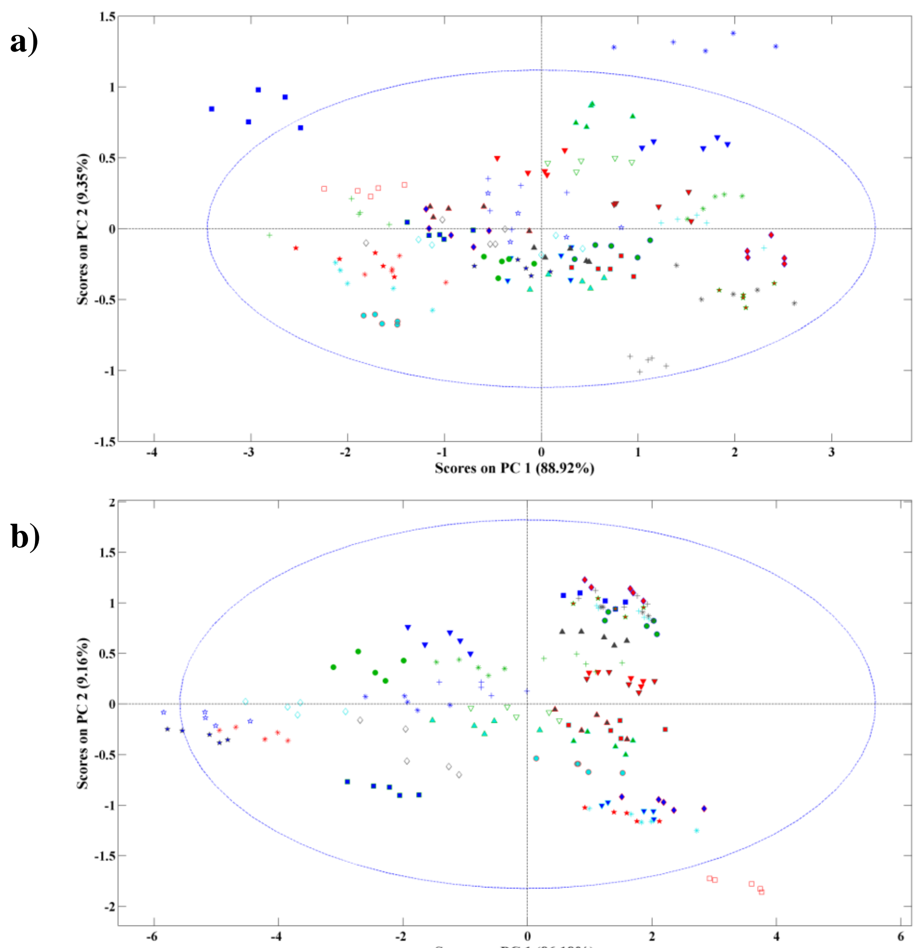


Figure 3.3. Scores of the first two components using all NIR (a) and MIR (b) spectra (mean centred). Legend: ▼ FUG; ✳ ELL; ■ NUG; + EKU; ◇ HTR; ▲ CAS; ★ SUM; ● NSN; ▼ MTH; ✳ CIT; □ CLU; + CRY; ◆ TOM; ▲ SGB; ★ BRX; ● BGO; ▽ CHA; ✳ GOL; ■ MOS; + HMG; ◆ WIL; △ TRG; ☆ NBR; ▼ SIM; ✳ SSE; ■ SAZ; + HEB; ◇ PER; ▲ VG1; ★ HAL; ● CHI; ▼ EKG; ✳ TET.

3.3.2.1.Spectral regions and pre-processing methods optimization

The NIR and MIR data were divided in five spectral regions as mentioned in the material and methods section with the objective of identifying spectral regions more informative in terms of the hop's variety. Several pre-processing techniques were tested as well. The rationale for selecting spectral windows and pre-processing methods was to minimise the distance within varieties while maximising the distance between varieties. The PLS-DA results obtained with the calibration set in terms of correct prediction percentages were used to determine the best spectral regions and pre-processing methods. The best pre-

processing technique for both NIR and MIR spectra was SNV followed by Savitzky-Golay with 15 points of filter width, 2nd polynomial order and 2nd derivative. The best spectral regions were: 6,079 to 5,389 cm^{-1} and 1,880 to 600 cm^{-1} for NIR and MIR technique, respectively.

3.3.2.2. Optimized models

After defining the best spectral region and pre-processing methods the first two principal components were plotted again (Figure 3.4). It is clear that after this optimization step, most samples are now clustered according to the variety, with some exceptions, for both NIR and MIR data. Looking with more detail for the NIR data (Figure 3.4a), NSN clustered very close to VG1, CIT with SUM and HTR with HAL. The hop varieties ELL, EKG, FUG and GOL clustered far apart from the rest. Regarding MIR data (Figure 3.4b), the varieties that clustered very close were: NSN with VG1, HAL with SAZ and CHA with HTR. HEB and CRY clustered far apart from the others. These findings indicate that both NIR and MIR spectral data contain information related to hop’s variety and suggest the application of a supervised classification method.

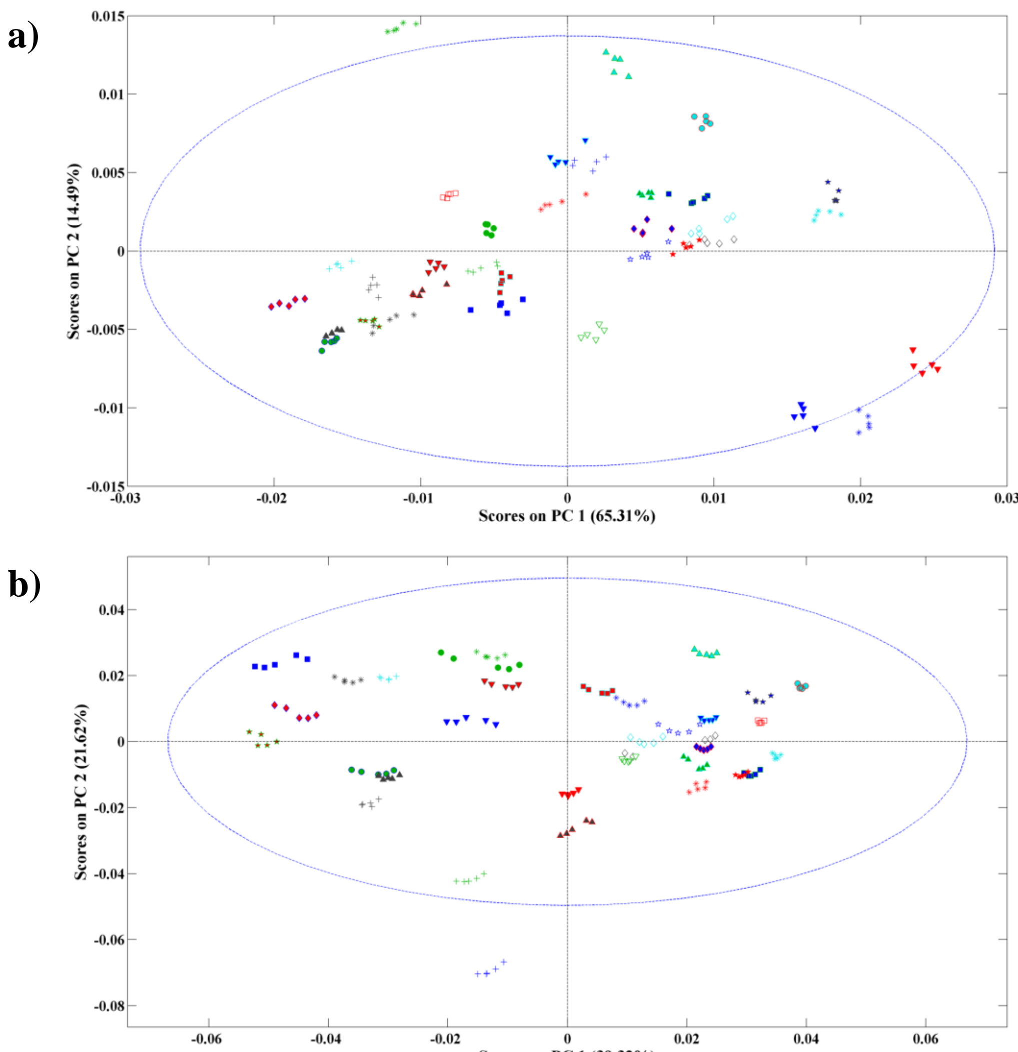


Figure 3.4. Scores of the first two components using the spectra pre-processed with SNV followed by Savitzky-Golay with 15 points of filter width, 2nd polynomial order and 2nd derivative and best spectral regions for NIR (a) and MIR (b) spectra. Legend: ▼ FUG; ✳ ELL; ■ NUG; + EKU; ◇ HTR; ▲ CAS; ★ SUM; ● NSN; ▼ MTH; ✳ CIT; □ CLU; + CRY; ◆ TOM; ▲ SGB; ★ BRX; ● BGO; ▽ CHA; ✳ GOL; ■ MOS; + HMG; ◆ WIL; △ TRG; ☆ NBR; ▼ SIM; ✳ SSE; ■ SAZ; + HEB; ◇ PER; ▲ VG1; ★ HAL; ● CHI; ▼ EKG; ✳ TET.

3.3.3. Unsupervised classification

Hierarchical cluster analysis (HCA) was applied for both NIR (Figure 3.5) and MIR (Figure 3.6) data considering the average of the five spectra of each hop variety and the best wavenumber region and pre-processing techniques as identified before. The dendrogram obtained using NIR data (Figure 3.5) grouped the hop varieties in two distinct clusters (C1

and C2). This distribution can be associated with the α-acids amount, because most of the hop varieties that contain a high amount of α-acids are clustered in C2 while varieties with α-acids content lower than 6% are aggregated in C1 cluster. The most similar hop varieties were Citra and Summit, and this is in agreement with the PCA findings. Regarding the dendrogram obtained using MIR data (Figure 3.5), the hop varieties were also grouped in two different clusters (C1 and C2). This distribution can also be correlated with α-acids amount considering that most of the hop varieties that contain a high amount of α-acids are aggregated in one cluster (in this case C1) but not so clearly as in the case of NIR spectroscopy.

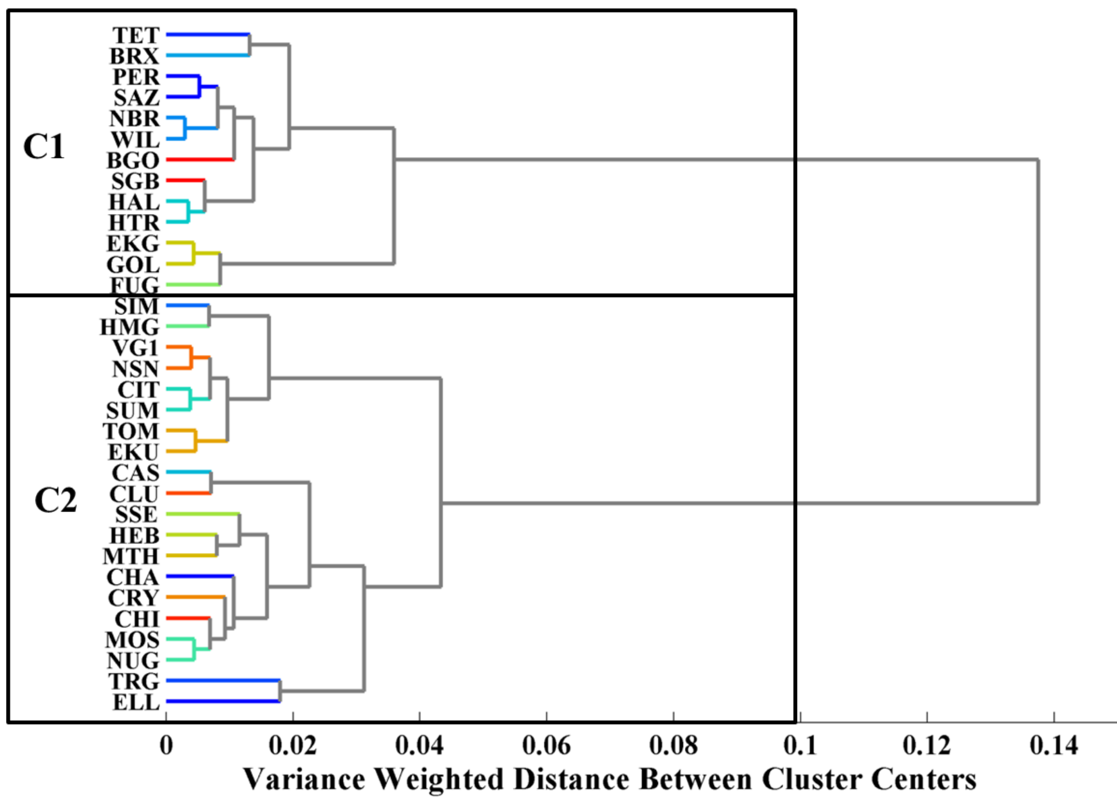


Figure 3.5. Dendrogram obtained with hierarchical cluster analysis (Ward's method) of commercial hops using NIR data between 6,079 and 5,389 cm^{-1} and pre-processed with SNV followed by Savitzky-Golay with 15 points of filter width, 2nd polynomial order and 2nd derivative.

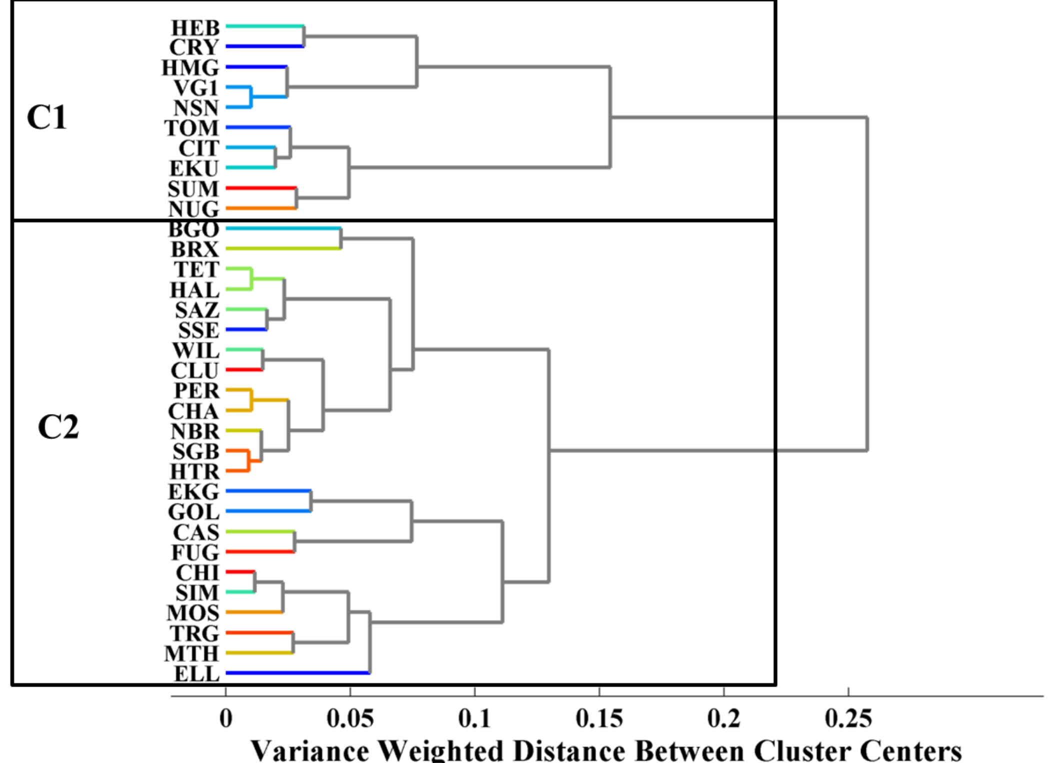


Figure 3.6. Dendrogram obtained with hierarchical cluster analysis (Ward's method) of commercial hops using MIR data between 1,880 to 600 cm^{-1} and with the best wavenumber region and pre-processed with SNV followed by Savitzky-Golay with 15 points of filter width, 2nd polynomial order and 2nd derivative.

3.3.4. Supervised classification

The selected supervised classification method used was PLS-DA. The test set of NIR and MIR data was then projected using the best spectral region and pre-processing technique. Models considering the NIR and MIR data obtained a total of 96.6% (8 LV) and 94.2% (10 LV) of correct predictions, respectively. The results demonstrate the applicability of both NIR and MIR techniques to discriminate the 33 hop varieties used in the present study in a non-destructive way which configures a useful tool for authenticity purposes. These results were obtained with only 5 samples per hop variety. Further studies should be made increasing the amount of different samples per variety and also using more varieties representing the cultivated plant full diversity.

The confusion matrices (one for each vibrational technique) revealed that there was no hop variety that could not be identified or misclassified with another hop (Supplementary

Table 2 and 3). In both confusion matrices the elements that are zero were not shown. The confusion matrix for NIR spectroscopy (Supplementary Table 2) revealed that the worst hop variety prediction involved PER and GOL with 74% and 79% of correct predictions, respectively. The hop variety PER was incorrectly predicted as NBR and SAZ while GOL was incorrectly predicted as FUG. Regarding MIR spectroscopy, the worst hop variety prediction involved SIM and PER with 79% and 72% of correct predictions, respectively. SIM was incorrectly predicted with MOS while PER was incorrectly predicted as NBR and SGB. The verified similarity between PER and NBR varieties (corresponding to the lowest number of correct predictions for both NIR and FTIR data) is also supported by the genetic data previously published using RAPD analysis (172) where authors describe the varieties as very close genetically, as well as the breeding history of the variety PER which has the NBR as the female parent (173). This similarity was verified as well in data concerning the SNP analysis performed for the varietal authenticity check.

Globally, the results obtained show the ability and accuracy of both NIR and MIR spectroscopic techniques to discriminate between different hop varieties.

3.3.4.1.Models analysis

The first four latent variables loadings obtained in the PLS-DA models using NIR and MIR data were depicted in Figure 3.7. These variables of the PLS-DA model using NIR data (encompassing approximately 96% of the total variance) revealed that the most important wavenumbers region was located between 6,000 and 5,600 cm^{-1}. This region corresponds to CH_3, CH_2, CH, SH and Ar-CH absorption bands (first overtone region). There is no published work that connects these absorption bands with the chemical compounds present in hop. Therefore, further studies should be performed in order to understand which chemical compounds are responsible for these absorption bands. However, once hops are rich in a complex mixture of polyphenolic compounds including, prenylphloroglucinols (α- and β- acids) and flavonoids (proanthocyanidins, flavanol glycosides, prenylchalcones and prenylflavanones) compounds can be related with the referred absorptions bands, together with a large fraction of volatile compounds, including terpenes (as myrcene, humulene, caryophyllene and farnesene among others) and molecules containing sulphur (as thiols, namely 4-mercapto-4-methyl-pentan-2-one, 3 mercaptohexan-1-ol, among others) (25, 41). This is supposed once the total of α-acids and β-acids can vary from low values as 5% to values over 20% of total dry weight depending on the hop variety (2). Moreover, the flavonoids fraction can vary between 3% and 6% (w/w), highlighting that the amount of the prenylchalcone xanthohumol, the major constituent of this group, can also vary from 0.1%

to 1% (w/w) (2, 54). The quantity of total oil is also known to vary considerably from 0.5% to 3.0% (v/w) among the different varieties of hops (174).

The first four latent variables of the PLS-DA model using MIR data (encompassing approximately 75% of the total variance) showed that the most important wavenumbers regions were located between 1,800 and 1,600 cm^{-1} and 1,400 and 1,000 cm^{-1}. The first region can be associated with proteins while the second can be connected with carbohydrates and DNA/ RNA/ phospholipids (167). In fact, another important parameter that substantially changes between hop varieties is the amount of proteins referenced to be between 12 to 22% dry matter (161) which can contribute to hops discrimination using MIR.

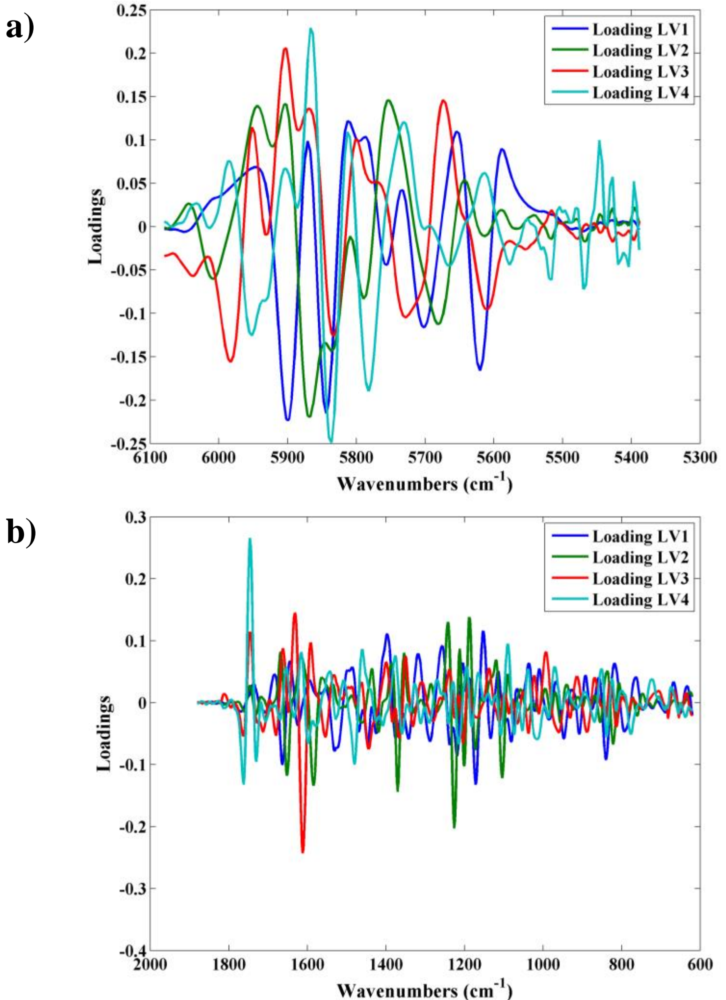


Figure 3.7. Representation of the loadings of the first four latent variables of the PLS-DA models using NIR (a) and MIR data (b).

3.3.5. Portuguese hops

Portuguese native hops were analysed by NIR, since it was considered better than MIR to discriminate between hop varieties. The model obtained a total of 89.9% (15 LV) of correct predictions. HCA (Figure 3.8) highlights three clusters. Two of them (C1, green and C2, blue) include the majority of Portuguese hops, which were grouped with the commercial varieties that present low content of α-acids. Concerning cluster C3 (red) it was formed by the varieties that present higher contents of α-acids together with 5 Portuguese hops (coded as PTG38, PTG53, PTG62, PTG63, and PTW7). PTW7 presented high content of α-acids (10.6 ± 0.2%), therefore it was correctly clustered. Nevertheless, the other four samples presented low to very low content of α-acids, (< 3.0%) though they were grouped in this cluster (Table 3.2).

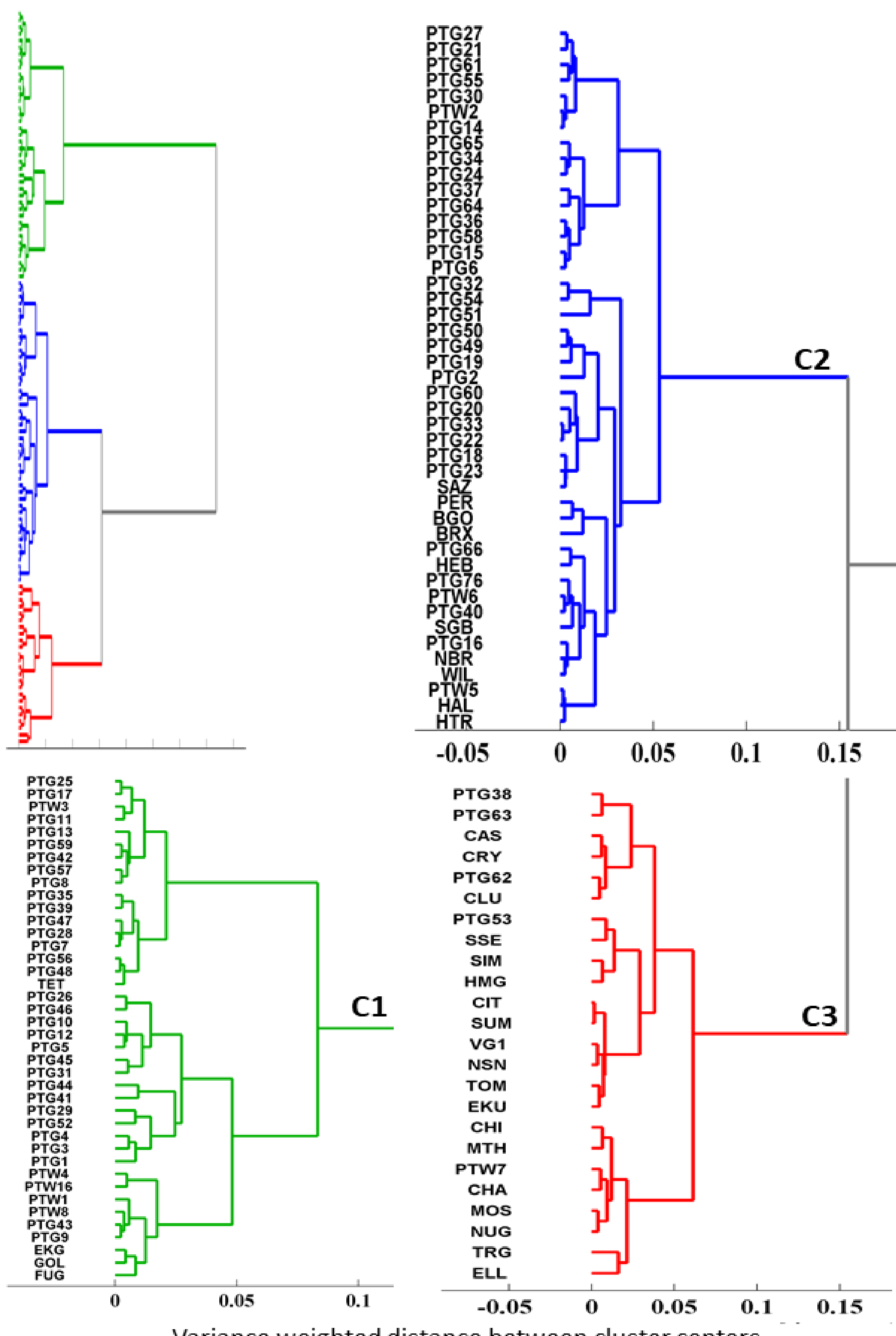


Figure 3.8. Dendrogram obtained with hierarchical cluster analysis (Ward's method) of Portuguese native hops and commercial varieties using NIR data between 6,079 and 5,389 cm^{-1} and pre-processed with SNV followed by Savitzky-Golay with 15 points of filter width, 2nd polynomial order and 2nd derivative. The three main clusters were separately highlighted.

Table 3.2. Xanthohumol (XN), α-acids, and β-acids of Portuguese native hops. All compounds are expressed in g/100 g of dryed hops (%).

Portuguese samples	XN (%)	α-acids (%)	β-acids (%)	Portuguese samples	XN (%)	α-acids (%)	β-acids (%)
PTG1	0.09	2.01	0.69	PTG39	0.09	0.56	1.55
PTG2	0.18	2.83	1.23	PTG40	0.12	1.63	1.44
PTG3	0.04	1.03	0.40	PTG41	0.03	0.00	0.55
PTG4	0.06	1.09	0.53	PTG42	0.08	1.24	1.72
PTG5	0.10	1.45	1.15	PTG43	0.49	1.11	1.61
PTG6	0.11	1.43	1.66	PTG44	0.02	0.20	1.44
PTG7	0.08	0.90	0.92	PTG45	0.06	0.30	1.85
PTG8	0.15	0.08	1.53	PTG46	0.05	0.50	0.94
PTG9	0.09	0.52	2.15	PTG47	0.15	1.57	1.52
PTG10	0.02	0.17	1.26	PTG48	0.34	0.82	1.43
PTG11	0.11	0.20	1.69	PTG49	0.16	1.31	1.94
PTG12	0.03	0.28	1.06	PTG50	0.08	1.54	1.56
PTG13	0.08	0.21	1.99	PTG51	0.05	0.26	1.71
PTG14	0.54	1.13	1.92	PTG52	0.06	0.20	1.02
PTG15	0.33	0.83	1.42	PTG53	0.41	1.35	1.48
PTG16	0.63	1.30	1.05	PTG54	0.04	0.04	1.23
PTG17	0.48	0.71	1.11	PTG55	0.22	0.20	2.19
PTG18	0.57	1.38	1.72	PTG56	0.32	0.65	1.56
PTG19	0.19	2.19	2.29	PTG57	0.21	0.20	0.97
PTG20	0.05	0.16	1.37	PTG58	0.46	0.91	1.75
PTG21	0.11	0.20	2.09	PTG59	0.47	0.71	1.46
PTG22	0.09	1.05	1.78	PTG60	0.10	1.82	1.89
PTG23	0.78	1.48	1.41	PTG61	0.05	0.29	1.84
PTG24	0.36	1.09	2.21	PTG62	0.20	0.20	1.79
PTG25	0.10	0.84	1.51	PTG63	0.06	0.36	1.82
PTG26	0.04	0.13	1.56	PTG64	0.04	0.19	1.69
PTG27	0.08	0.35	1.38	PTG65	0.13	1.84	1.80
PTG28	0.07	0.48	1.43	PTG66	0.08	0.34	2.08
PTG29	0.04	0.12	1.04	PTG76	0.15	1.86	1.65
PTG30	0.09	1.00	1.67	PTW1	0.14	3.12	3.58
PTG31	0.03	0.20	1.21	PTW2	0.22	2.65	6.31
PTG32	0.11	0.63	1.45	PTW3	0.00	1.46	1.31
PTG33	0.05	0.40	2.03	PTW4	0.00	1.22	0.91
PTG34	0.55	1.53	1.59	PTW5	0.41	5.84	4.08
PTG35	0.08	0.15	1.41	PTW6	0.41	6.09	4.30
PTG36	0.29	0.65	1.31	PTW7	0.51	10.59	3.15
PTG37	0.01	0.65	1.81	PTW8	0.31	5.28	3.99
PTG38	0.10	0.89	2.58	PTW16	0.09	2.87	2.10

NIR spectroscopy is based in vibrational detection of C–H, N–H, O–H, and S–H bonds. Once present in much higher concentration, the α-acids could be interpreted as the most important compounds for these analyses. But, in the case of hops with low content of α-acids, it is possible that other compounds also influence NIR analysis. After α-acids, in general, β-acids is considered the second most abundant fraction in hops and could influence these results, however, neither the data from β-acids content explain the

clustering generated. The same conclusion could be reached with the results from xanthohumol content. Probably, other metabolites were involved. However, it should be highlighted that with commercial hops, HEB and SSE that present α-acids content 2.3% and 5.2%, similar situation occurred, because they clustered together with those that contained higher than 6 % of α-acids.

3.4. Conclusion

This work assesses the suitability of NIR and MIR spectroscopy to discriminate hop genotypes. The 33 commercial varieties analyzed represent the majority of the hops world market (about 75%) which assures the applicability of the present method as a rapid and non-destructive tool that can be used for authenticity purposes in the hops supply chains. The increasingly high value of hop elite varieties can promote fraudulent practices in the market which sharpens the need of better and more expedite authenticity tools like the one herein proposed.

Unsupervised methods applied to spectra provided indications that these techniques can be able to discriminate between hop varieties and suggested the application of a supervised method. After optimising the wavenumbers region and pre-processing techniques, a total of 96.6% (8 LV) and 94.2% (10 LV) of correct hop varieties prediction were obtained for NIR and MIR spectroscopy, respectively. Additionally, dendrogram obtained using NIR data was able to group the samples according to the α-acids content, one cluster for high amount of α-acids (>6%) and another cluster for the varieties that present lower α-acids content. Therefore, discrimination of 33 commercial and 75 Portuguese native hops was done by NIR, and 89.9% (15 LV) of correct hop varieties prediction were obtained. The results obtained in this work are very promising and demonstrate the suitability of both vibrational techniques. Moreover, these methods are rapid, cost-effective, non-destructive and environmentally friendly (do not generate residues). Further studies including more samples per variety and reflecting different geographical origins, when possible, and years of harvest are needed to attest the robustness of these techniques.

CHAPTER 4

Volatile and sensory characteristics of Portuguese native hops: comparison with commercial varieties

4.1. Introduction

The noble hop aromas are associated with the essential oil fraction that contains a complex group of volatiles, about 440 compounds were already identified (2), although, it was suggested that more than one thousand volatile compounds can be found (175). This fraction includes a highly diverse group of esters and terpenes, and also, in smaller amounts, aldehydes, aliphatic hydrocarbons, carboxylic acids, furans, higher alcohols, ketones, phenols and sulphur compounds (2). Usually, studies concerning the volatile profile of hops are carried out after extraction of essential oils, nonetheless the analysis in spontaneous volatile emission provides a more realistic insight, closer to the brewing practices, specifically in the case of dry-hopped beers, where the hop is added at cold stages of the production (1). The spontaneous volatile emission profile can be analysed through headspace solid-phase microextraction combined with mass spectrometry and chemometrics, which has been a powerful analytical tool for profiling the hop volatile metabolomic pattern, and also for description and discrimination of hop varieties (57-59, 111, 176). Moreover, hops sensory evaluation is of major relevance to characterize hop samples concerning specific aroma attributes (13, 123, 124).

The aim of this study was the characterization of volatile fraction of Portuguese hops, and comparison with 34 commercial varieties regarding: (i) spontaneous volatile emission profile by headspace analysis of hop inflorescences; and (ii) aroma attributes, using a semi-trained panel for sensory evaluation. Moreover, relationships between volatile profile and aroma attributes collected directly from hops were explored. Chemometric techniques were applied, in order to analyse all data collected, using mathematical statistics, since it provides valuable information and facilitates the detection of hidden relationships between variables.

4.2. Materials and methods

4.2.1. Chemical and materials

Reference standards methyl nonanoate (≥ 99.8%), citral (≥ 95%), *cis*-3-hexen-1-ol (≥ 99.9%), (+)-β-pinene (≥ 98.5%), furaneol (≥ 99%), linalool (97%), and myrcene (≥ 90%) were purchased from Sigma Aldrich (St. Louis, Mo., US).

4.2.2. Hop samples

The majority hops samples used in chapter 3 were analyzed for volatile and sensory characterization. The exceptions were the addition of a commercial variety (PLA) and two Portuguese genotypes (PTG76 and PTW16), and the exclusion of PTW09. Thus, a total of 109 hop samples (harvest 2016) were analyzed: 75 Portuguese native and 34 commercials hops (Supplementary Table 1).

4.2.3. Headspace-solid phase micro-extraction (HS-SPME) coupled to gas-chromatography mass-spectrometry

Grounded samples (0.5 g) placed in 10 ml vials with polypropylene caps were equilibrated for 30 min at 40 °C. After this equilibration period, a fibre 100 µm PDMS was exposed to the upper space of the sample for 15 min at 40 °C (177).

Gas-chromatographic analysis mass-spectrometry (GC-MS) was performed on an Agilent 6890 series GC (Agilent, Avondale, PA, US), with splitless injection, coupled to a MS detector (Agilent 5973). Volatiles were separated using a bonded phase fused-silica capillary column (SPB-5, 60 m × 0.32 mm × 1 µm, Supelco, Bellefonte, US), operating at constant flow with helium at 1.2 ml min^{-1}. Hops samples extracted were desorbed with an injection port at 250 °C/ 2 min in splitless mode. For the chromatographic separation, the column temperature starts at 40 °C, held for 1 min, increased at 1 °C/ min to 90 °C and at 2 °C/ min from 90 to 140 °C, then the temperature was raised to 250 °C, at rate of 40 °C/ min and held for 1.25 min. Samples were analysed in full-scan mode, from 50 to 550 m/z and the analytes were identified by the retention time confirmed by NIST (National Institute of Standards and Technology, US) library database.

4.2.4. Sensory evaluation

A panel composed of 16 participants (20–50 years of age), including students and employees from University of Porto, was initially recruited on the basis of interest and availability. The panel underwent a partial training for hops attributes, including two training sessions (approximately 1 h each), which were focused on providing a clear definition of the attribute list, used in the check-all-that-apply (CATA) ballot. The first session included training attributes by the use of standard references (Table 4.1), whereas the second session training was conducted with hop and attribute references.

After the training session, the panel was able to evaluate a list of six aroma attributes commonly used to characterize hops: “Citrus”, “Fruity/Sweet”, “Floral”, “Spicy”, “Resinous”, and “Herbal”. CATA assay was also explained to participants during training.

Table 4.1. Attributes list used in CATA ballots, along with hop/standard and attribute references provided to semi-trained participants during training

	Attributes description	Hop references	Standard references[1]	Attribute references
Citrus	Orange, lemon, tangerine	CIT	Citral and linalool	Citric fruits peel (orange, lemon, tangerine and grapefruit)
Floral	Flowers	ELL	Linalool	Lawn flowers petal
Fruity	Melon, banana, papaya, mango, green apple, berries	MOS	Methyl-nanonoate	Pieces of not-citric fruits (melon, green apple and berries)
Herbal	Grass, leaves, hay, tea	FUG	*cis*-3-hexen-1-ol	Cut grass
Resinous	Resin, cedar, pine	CAS	(+)-β-Pinene	Shell and resin of oak
Spicy	Pepper, curry, clove, coriander seeds	HTU	Myrcene	Ground peppers
Sweet	Caramel	HAL	Furaneol	Caramelized sugar

Sensory evaluation was performed individually under white light at room temperature. The definition spreadsheet with the personal notes was available to participants throughout evaluation sessions. Samples were blind-labelled with a three-digit code and the serving order of samples was randomized and balanced to account for first order and carry-over effects (178).

4.2.5. Data pretreatment

Data pretreatment includes normalization and scaling, which is a required process for data that present wide scale differences, as is the case of volatiles (179). Normalization treatments are applied to each sample, using a correcting factor (e.g., total area) that adjusts the peak intensities and reduces unwanted drifts between samples, allowing their comparison (180, 181). Scaling is applied to each variable/volatile compound and adjusts the importance of each variable with a scaling factor, altering the relative distance between variables (180). Data scaling includes statistical methods such as Pareto scaling, auto

scaling (also known as Z-score), and centring. This converts data to fluctuations around zero, subtracting the mean of a variable to the original values. Pareto and auto scaling apply centring but also add a scaling factor (standard deviation and square root of the standard deviation, respectively). Auto-scaling was selected as it had the highest ability to distinguish between commercial and Portuguese hop samples.

4.2.6. Data reduction

In order to validate the reduction of volatile compounds analyzed, a comparison of multivariate patterns was carried out through Procrustes analysis (Figure 4.1). For this, the first 10 principal components score plots were used for comparison, with data obtained from 109 volatile compounds (X) being used as the reference and data obtained from 32 volatile compounds (Y) as the one to be manipulated. Hence, Y data was manipulated in order to resemble X data using three transformations: reflection, rotation, and translation (Z). The goodness-of-fit criterion of this analysis is the minimized sum of squared errors (dissimilarity between X and Y), which varies between 0 (X and Y are similar) and 1 (X and Y are dissimilar). As the observed value of dissimilarity between X and Y plot was 0.2635, the data from the selected 32 compounds could be used without compromising relevant information. For this reason, the selected 32 compounds were used for further analysis.

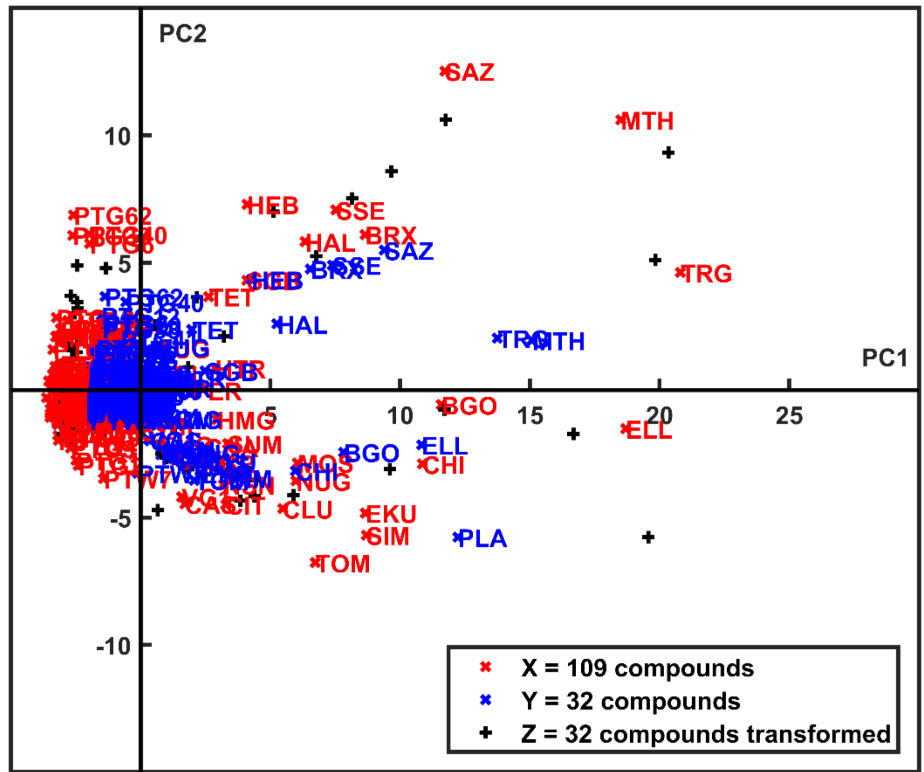


Figure 4.1. Hop samples comparison of score plots for 109 and 32 compounds.

4.2.7. Statistical analysis

All volatile compounds analyzed were tested for distribution of the residuals with Shapiro–Wilk's test. For each compound, mean or median values of the sample triplicates were used, according to normal or non-normal distribution of the residuals.

The data obtained from volatile composition was normalized and scaled through pre-treatment processing described in the previous section. The distribution of the 109 hop samples with 109 and 32 volatile compounds was analysed by Agglomerative Hierarchical Clustering (AHC) analysis and then displayed as a cluster heatmap. Different methods of hierarchical clustering and distance between samples were tested to produce AHC dendrograms (tree). Principal Components Analysis (PCA) was conducted with both variable selections and procrustes analysis was carried out to compare principal component score plots and assess similarity between both variable selections. Partial least squares discriminant analysis (PLS-DA) was conducted to develop models to find a two dimensional

planes (discriminating planes) in which commercial and Portuguese hop samples (projected observations) on the PLS components were well separated according to their volatile compounds. A selection of volatile compounds that presented statistical significances between Portuguese and commercial hop samples was carried out prior to PLS-DA. These volatile compounds were identified through the one-way analysis of variance (ANOVA) or Kruskal–Wallis, depending on normal distribution of the residuals was confirmed or not. Random validation was also applied to identify relevant X-variables. Scores and loading plots were analysed, as well as, calibration and validation coefficients.

For an overall test of CATA data, Cochran's Q test (182) was performed to determine whether the proportions of selection by the semi-trained panel for individual attributes of the CATA question differed as a function of hop sample and type (commercial or Portuguese). If there was a significant difference among the variables, post hoc multiple pairwise comparisons were performed using Marascuilo's. In addition, correspondence analysis, based on chi-square distance, was used to visualize associations between the CATA attributes and the hop samples. Significant terms determined by Cochran's Q test were applied for correspondence analysis.

Furthermore, PLS regression was also used to study the relationships between sensory aroma (Y-matrix) and volatile composition (X-matrix) of hops in terms of prediction of Y-variables from X-variables. Random validation was also applied to identify relevant X-variables. Scores and loading plots were analysed, as well as, calibration and validation coefficients.

All analyses were performed at 5% significance level. Heatmap plots and AHC were carried out using the heatmap.2 function from the gplot package in R (183). PCA was performed using the factoextra package (184) and FactoMineR package (185) in R. Procrustes analysis was carried out using the Statistics and Machine Learning Toolbox available with Matlab R2016a (MathWorks, Natick, US). Partial least squares-discrimination analysis (PLS-DA), Pearson´s correlation and PCA were performed using XLSTAT® for Windows version 2016.02 (Addinsoft, Paris, France).

4.3. Results and discussion

4.3.1. Volatile fraction characterization

4.3.1.1.Headspace of dried cones and chemometric analysis

Agglomerative hierarchical clustering (AHC) with heatmap

A total of 109 compounds across all 109 hop samples were identified. A preliminary overview of hop samples was obtained using agglomerative hierarchical clustering (AHC) analysis, which was applied for all the 109 compounds identified. AHC was also applied to a selection of 32 compounds that are reported in literature as odour-active compounds.

AHC is an unsupervised chemometric technique that reveals the natural groupings existing between samples characterized by the values of a set of measured variables. The similarities between samples were analysed using different distance measures and linkage methods, which were evaluated through the cophenetic correlation coefficient (CPCC). CPCC is a measure of how faithfully a dendrogram preserves the pairwise distances between the original unmodeled data points. The Euclidean distance and average linkage were selected to establish the clusters (CPCC = 0.981, for 109 compounds; CPCC = 0.965, for 32 compounds).

A cluster heatmap was used to visualize the results obtained from AHC, revealing dendrogram structure and cluster trends within the hop samples and volatile compounds (Figures 4.2 and 4.3, 109 and 32 compounds respectively). The cluster heatmap consists of a rectangular tiling, with each tile shaded on a colour scale to represent the value of the corresponding element of the data matrix. In this case, blue represents higher intensities, and red represents low intensities.

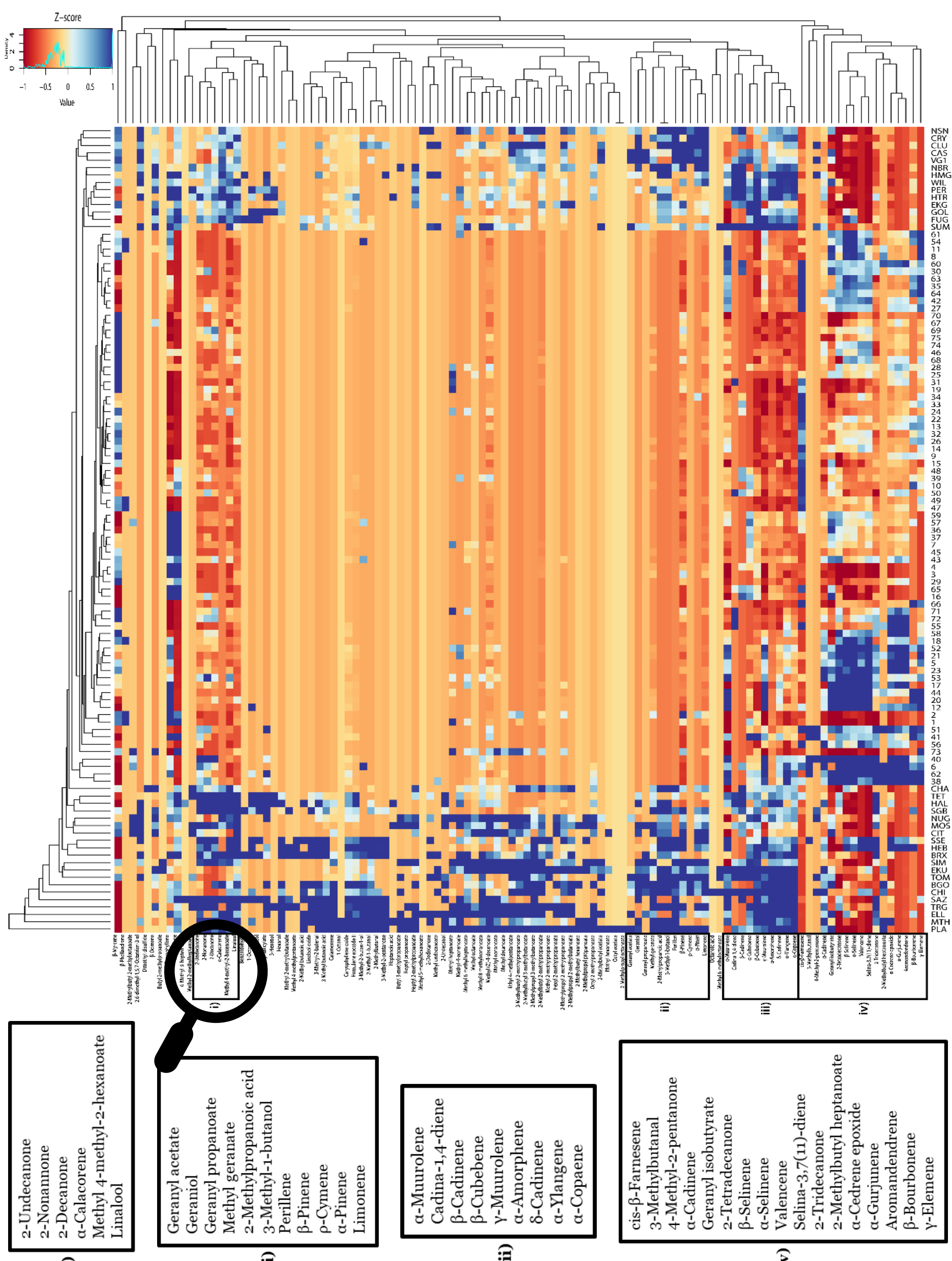


Figure 4.2. Heatmap of all 109 volatile compounds tentatively identified in hop samples. Blue colour represents higher intensities, while red represents lower intensities. Different groups of compounds with similar pattern in Portuguese native and commercial hops were identified, from (i) to (iv). Legend: 1 to 66, PTG1 to PTG66; 67 to 75, PTW1 to PTW9.

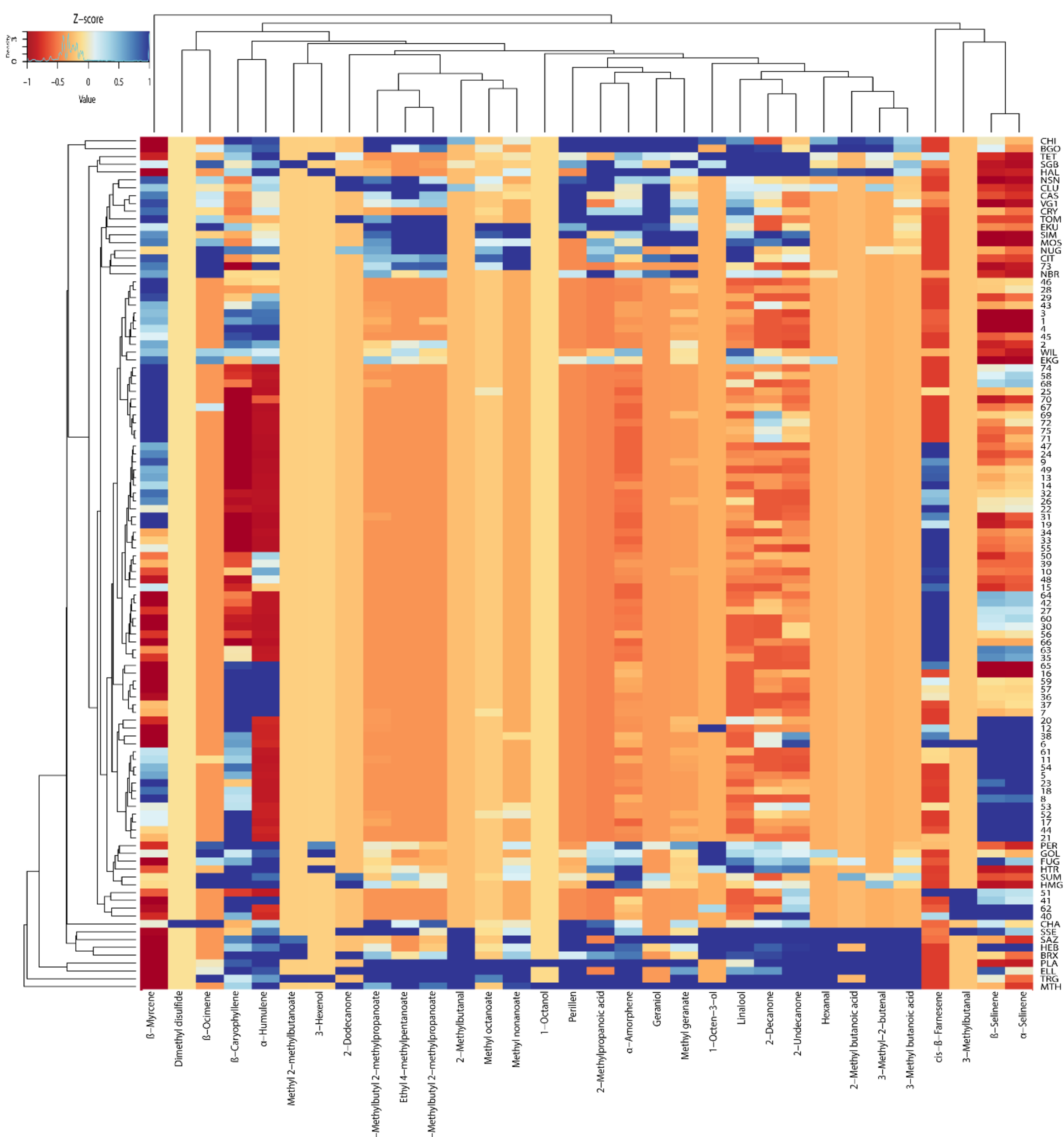


Figure 4.3. Heatmap of selected 32 volatile compounds tentatively identified in hop samples. Blue colour represents higher intensities, while red represents lower intensities. Legend: 1 to 66, PTG1 to PTG66; 67 to 75, PTW1 to PTW9.

It is possible to observe different profiles between commercial and Portuguese hops. In analyses with 109 compounds the majority of commercial hops were dispersed, except a group formed by NSN and CRY, other by CAS and VG1, and a group with 9 hops, NBR, HMG, WIL, PER, HTR, EKG, GOL, FUG and SUM. Portuguese genotypes were grouped in

a big cluster that included 69 hops, and only 6 were separated into two different clusters (PTG56, PTW7, PTG40 and PTG6 in one, and another cluster with PTG62 and PTG38).

In the heatmap presented in Figure 4.2, 3 groups of compounds (i, ii and iii) that present lower percentage in Portuguese hops, when compared with the commercial ones, were highlighted: (i) includes Linalool and the ketones 2-undecanone, 2-decanone, and 2-nonanone, considered important to citric, fruity and floral notes; (ii) geraniol and other floral compounds; and typical compounds with strong herbal/ terpenic characteristics, such as α- and β-pinene; and (iii) a third group formed only by terpenic compounds. An opposite trend was observed for another group of compounds (iv), which included some terpenes with woody organoleptic characteristics, particularly cis-β-farnesene (woody), and some esters, aldehydes, and ketones that, in general, showed lower percentage in commercial samples than Portuguese hops.

β-myrcene (spicy), β-caryophyllene (woody) and α-humulene (woody) demonstrated a variable pattern, from very low to very high percentage for both commercial and Portuguese hops.

In contrast to the wide number of volatile compounds that have already been identified in hops, only few of them can be detected by human noses. This has been demonstrated by several studies that coupled gas chromatography and olfactometry assays (31, 60-62, 64, 68, 121, 122, 186, 187). From the 109 compounds identified in this work, 32 were already cited as odour-active (Table 4.2). The analyses of this selection with 32 compounds showed great similarities with the one using 109 (Figures 4.2 and 4.3), namely a clear distinction between commercial and Portuguese hops; furthermore, it was possible to have more samples included in clusters. Some peculiarities were also observed, namely i) the varieties NSN, CRY, CAS, VG1 are in the same cluster, together with CLU and TOM; ii) there is a new cluster with only SIM and MOS; iii) PER, GOL, FUG, HTR, SUM and HMG remain together in the same group, however, WIL and EKG appear in the cluster of Portuguese hops, and the wild PTW7 was in a cluster with NBR, NUG and CIT; iv) the Portuguese samples PTG62/ PTG40 and PTG51/ PTG41 were separated from the group of Portuguese hops and were included in two different clusters.

Table 4.2. Retention time, odour type/ description in pure compounds and sniffing impression described by Olfactometry in hops.

	RT[a]	Odour type: description [b]	GC-O in hops[c]
Aldehydes and ketones			
3-Methylbutanal	8.66	Aldehydic: ethereal, aldehydic, chocolate, peach, fatty	sweet, malty
2-Methylbutanal	8.78	Chocolate: musty, chocolate, malty, fermented, cocoa, coffee, nutty	sweet, malty
4-Methyl-2-pentanone	11.63	Green: sharp, solvent, green, herbal, fruity, dairy, spicy	
3-Methyl-2-pentanone	12.28	n.f.	
3-Methyl-2-butenal	13.79	Fruity: sweet, fruity, pungent, brown, nutty, almond cherry	almond, roasted
Hexanal	14.66	Green: fresh, green, fatty, aldehydic, clean, grassy, leafy, vegetable, fruity, sweaty	green, leafy, sweet, unpleasant
Benzaldehyde	28.27	Fruity: sharp, sweet, bitter, almond, fruity, powdery, nutty, cherry, maraschino cherry	
6-Methyl-5-hepten-2-one	30.55	Citrus: citrus, green, musty, fruity, lemongrass, apple, ketonic, creamy, cheesy, banana	
2-Nonanone	43.24	Fruity: fresh, fruity, sweet, waxy, soapy, cheesy, green, weedy, earthy, herbal, coconut	
2-Decanone	55.91	Floral: orange, floral, fatty, peach	earthy, moldy, musty
2-Undecanone	61.04	Fruity: waxy, fruity, pineapple, creamy, ketonic, fatty, orris, floral	floral, citrus
2-Dodecanone	62.98	Citrus: fruity, citrus, floral, orange	citrus
2-Tridecanone	65.91	Waxy: fatty, waxy, mushroom, dairy, milky, coconut, nutty, herbal, earthy, chicken, fat, fatty	
2-Tetradecanone	67.56	n.f.	
Alcohols			
2-Methyl-1-butanol	11.46	Roasted: roasted, winey, onion, fruity, fusel, alcoholic, whiskey	
3-Methyl-1-butanol	11.93	Fermented: fusel, alcoholic, pungent, ethereal, whiskey, cognac, fruity, banana, molasses	
3-Methyl-2-buten-1-ol	13.22	Fruity: sweet, fruity, alcoholic, green, lavender	
3-Hexenol	18.35	Green: green, leafy	green
1-Octen-3-ol	29.66	Earthy: mushroom, earthy, green, oily, vegetable, fungal, raw chicken	mushroom-like
1-Octanol	40.37	Waxy: waxy, green, citrus, orange, aldehydic, floral, rose, sweet, fatty, coconut, mushroom	citrus-like, soapy
2,6-dimethyl-1,5,7-Octatrien-3-ol	51.80	Camphoreous: camphoreous, lime	
2-Undecanol	61.26	Waxy: fresh, waxy, cloth, laundered, cloth, sarsaparilla	

[a] Minutes. [b] Odour types and descriptions of pure compounds found in http://www.thegoodscentscompany.com (188). [c] Odorant descriptors found in a comprehensive literature search (31, 60-62, 64, 68, 121, 122, 186, 187). n.f., not found.

Table 4.2. Retention time, odour type/ description in pure compounds and sniffing impression described by Olfactometry in hops (continued).

	RT[a]	Odour type: description [b]	GC-O in hops[c]
Terpenes and terpenoids			
α-Pinene	25.88	Herbal: herbal, fresh, cooling, camphoreous, sweet, pine, terpenic, earthy, woody	
β-Pinene	30.41	Herbal: dry, cooling, woody, resinous, pine, hay, terpenic, green, fresh, minty, eucalyptus, camphoreous, spicy, peppery, nutmeg	
β-Myrcene	31.46	Spicy: peppery, terpenic, herbal, spicy, woody, balsamic, plastic, rose, celery, carrot	fresh hops, citrus/greenery, lime, metallic, geranium-like
p-Cymene	35.53	Terpenic: chemical, woody, fresh, terpenic, citrus, lemon, spicy, cumin, origanum, cilantro	
Limonene	36.09	Terpenic: citrus, herbal, pine, terpenic, camphoreous	
β-Phellandrene	36.28	Minty: minty, terpenic	
β-Ocimene	37.95	Floral: citrus, tropical, green, terpenic, woody, vegetable	green, floral
Linalool	44.40	Floral: citrus, orange, floral, terpenic, sweet, rose, waxy, aldehydic, woody, green, blueberry	sweet, flowery, citrus-like, terpenic, fresh
α-Terpineol	56.16	Terpenic: terpenic, pine, woody, resinous, cooling, lemon, lime, citrus, floral, lilac	
Geraniol	59.53	Floral: sweet, floral, fruity, rose, waxy, citronella, citrus	floral, rose-like, geranium
(Z)-Methyl geranate	62.04	Floral: floral, herbal, citrus, fruity, green, geranium	greenery
α-Cubebene	63.12	Herbal: herbal, waxy	
Geranyl acetate	63.58	Floral: floral, rose, lavender, herbal, green, cooling, waxy	
α-Ylangene	63.81	n.f.	
α-Copaene	63.90	Woody: woody, spicy, honey	
β-Bourbonene	64.18	Herbal: herbal, woody	
α-Gurjunene	64.77	Woody: woody, balsamic	
β-Caryophyllene	65.04	Spicy: sweet, woody, terpenic, spicy, clove, dry	spicy
γ-Elemene	65.09	green, woody, oily	
β-Cubebene	65.17	Citrus: citrus, fruity, radish	
cis-β-Farnesene	65.26	Woody: woody, citrus, herbal, floral, lavender, bergamot, myrrh, neroli, green, vegetable	woody
Aromandendrene	65.44	n.f.	
Geranyl propanoate	65.49	Floral: floral, fresh, waxy, fruity, rose, honey, tropical, vegetable, powdery	
α-Humulene	65.74	Woody: woody	resin, balsamic
γ-Muurolene	66.02	Woody: herbal, woody, spicy	
α-Muurolene	66.09	Woody: woody	

[a] Minutes. [b] Odour types and descriptions of pure compounds found in http://www.thegoodscentscompany.com (188). [c] Odorant descriptors found in a comprehensive literature search (31, 60-62, 64, 68, 121, 122, 186, 187). n.f., not found.

Table 4.2. Retention time, odour type/ description in pure compounds and sniffing impression described by Olfactometry in hops (continued).

	RT[a]	Odour type: description [b]	GC-O in hops[c]
Terpenes and terpenoids (continued)			
Geranyl isobutyrate	66.18	Floral: sweet, floral, fruity, green, peach, apricot, rose	
Cadina-1,4-diene	66.31	Spicy: spicy, fruity, mango	
β-Selinene	66.37	Herbal	herbs, pungent
α-selinene	66.49	Amber	woody, pungent
β-Cadinene	66.57	Woody: green, woody	
α-Amorphene	66.72	n.f.	woody
δ-Cadinene	66.78	Herbal: thyme, herbal, woody, dry	
Calamenene	66.86	herb, spice	
Valencene	66.96	Citrus: sweet, fresh, citrus, grapefruit, woody, orange, dry, green, oily	
α-Cadinene	67.10	Woody: woody, dry	
α-Calacorene	67.23	Woody: woody	
Selina-3,7(11)-diene	67.30	n.f.	
Esters			
Methyl 2-methylpropanoate	9.54	Fruity: ethereal, fruity, sweet, tutti frutti, floral, apple, pineapple	
Methyl 2-methylbutanoate	13.34	Fruity: ethereal, estery, fruity, tutti frutti, apple, green apple, lily of the valley, powdery, ripe, fatty, green	sweet, fruity
2-Methylpropyl propanoate	19.15	Fruity: fruity, green, ethereal, sweet, tutti frutti, banana, rummy, pungent, bubble gum, estery, tropical	
2-Methylbutyl acetate	20.06	Fruity: fruit overripe, fruit sweet, banana, juicy fruit, fruity, sweet, ripe, estery, tropical	
Methyl 4-methylpentanoate	21.08	Fruity: fruity, sweet, banana, pineapple, cheesy	
2-Methylpropyl 2-methylpropanoate	23.13	Fruity: ethereal, fruity, tropical fruit, pineapple, grape skin, banana	
Methyl hexanoate	24.05	Fruity: fruity, pineapple, ethereal	
Butyl 2-methylpropanoate	26.85	Fruity: fruity, sweet, tutti frutti, green, melon, tropical, apple, banana, citrus, cheesy	
Butyl 3-methylpropanoate	28.57	Fruity: sweet, fruity, apple, raspberry, green, banana	
Ethyl 4-methylpentanoate	28.82	Fruity: fruity	sweet, fruity, citrus, pineapple
Methyl 5-methylhexanoate	30.76	n.f.	
2-Methylpropyl 2-methylbutanoate	32.42	Fruity: sweet, fruity	
2-Methylpropyl 3-methylbutanoate	32.71	Fruity: sweet, fruity, apple, raspberry, green, banana	

[a] Minutes. [b] Odour types and descriptions of pure compounds found in http://www.thegoodscentscompany.com (188). [c] Odorant descriptors found in a comprehensive literature search (31, 60-62, 64, 68, 121, 122, 186, 187). n.f., not found.

Table 4.2. Retention time, odour type/ description in pure compounds and sniffing impression described by Olfactometry in hops (continued).

	RT[a]	Odour type: description [b]	GC-O in hops[c]
3-Methylbutyl 2-methylpropanoate	33.48	Fruity: sweet, fruity, estery, waxy, apricot, pineapple, green, banana	fruity, spicy, sweet
2-Methylbutyl 2-methylpropanoate	33.90	Fruity: fruity, ethereal, tropical, banana	cooked vegetable
Methyl heptanoate	34.79	Fruity: sweet, fruity, green, orris, waxy, apple, floral, berry	
Methyl 4-methyl-2-hexenoate	35.28	n.f.	
Methyl 2-methylheptanoate	39.52	n.f.	
Methyl 6-methylheptanoate	42.60	n.f.	
2-Methylbutyl 2-methylbutanoate	44.88	Fruity: sweet, fruity, estery, berry, green, waxy, apple	
2-Methylbutyl 3-methylbutanoate	45.35	Fruity: herbal, fruity, earthy, sweaty, cheesy, apple, apple skin, green, winey, cognac	
Methyl octanoate	47.38	Waxy: waxy, green, sweet, orange, aldehydic, vegetable, herbal	fruity
Hexyl 2-methylpropanoate	50.52	Green: sweet, green, fruity, apple, pear, winey, grape, ripe, peach, berry	
Methyl 6-methyloctanoate	55.95	n.f.	
Methyl salicylate	56.48	Minty: sweet, root beer, wintergreen, minty, aromatic, phenolic, camphoreous	
Heptyl propanoate	56.70	Floral: rose, apricot	
Octyl acetate	57.16	Floral: green, earthy, mushroom, herbal, waxy, fruity, apple	
Methyl-4-nonenoate	57.28	n.f.	
Methyl nonanoate	57.97	Fruity: sweet, fruity, pear, waxy, winey, tropical, winey	floral, fruity, citrus
Heptyl 2-methylpropanoate	59.09	Fruity: sweet, green, fruity, warm, floral, estery, pineapple, apple, cherry, apricot, peach	
2-Methylbutyl hexanoate	59.41	Ethereal	
Methyl 8-methylnonanoate	60.78	n.f.	
Methyl (Z)-4-decenoate	61.54	Fruity: fruity, pear, mango, fishy, peach, green	
Methyl decanoate	61.96	Fermented: oily, winey, fruity, floral	
Octyl 2-methylpropanoate	62.55	Waxy: oily, green, waxy, soapy, aldehydic, clean, fruity, green, earthy, creamy	
2-Methylpropyl octanoate	62.64	Fruity: fruity, green, oily, floral	
2-Methylbutyl heptanoate	62.77	n.f.	
Methyl undecanoate	63.69	Waxy: fatty, waxy, fruity	
Furans			
Perillen			citrus
3-(4-methylpent-3-enyl) furan	44.71	Woody: woody	

[a] Minutes. [b] Odour types and descriptions of pure compounds found in http://www.thegoodscentscompany.com (188). [c] Odorant descriptors found in a comprehensive literature search (31, 60-62, 64, 68, 121, 122, 186, 187). n.f., not found.

Table 4.2. Retention time, odour type/ description in pure compounds and sniffing impression described by Olfactometry in hops (continued).

	RT[a]	Odour type: description[b]	GC-O in hops[c]
Carboxylic acids			
2-Methylpropanoic acid	11.93	Acidic: acidic, sour, cheesy, dairy, buttery, rancid	sweet, malty, cheesy
3-Methyl butanoic acid	16.35	Cheesy: cheesy, dairy, acidic, sour, sweaty, pungent, fruity, ripe fatty, tropical	goaty, sweaty, cheesy
2-Methyl butanoic acid	17.05	Acidic: pungent, acidic, fruity, dirty, fermented, cheesy, roquefort cheese	cheesy
Heptanoic acid	40.03	Cheesy: rancid, sour, cheesy, waxy, sweaty, fermented, pineapple, fruity	
Octanoic acid	48.31	Fatty: fatty, waxy, rancid, oily, vegetable, cheesy	
Others			
Dimethyl disulfide	12.16	Sulphurous: sulphurous, vegetable, cabbage, onion	solvent
Linalool oxide	41.29	Floral: woody, floral, cooling, terpenic, herbal, earthy, green	
α-Cedrene epoxide	65.56	Woody: woody, amber, tobacco, sandalwood, fresh, herbal, patchouli	
Caryophyllene oxide	68.04	Woody: sweet, fresh, dry, woody, spicy	
Humulene epoxide II	68.42	n.f.	

[a] Minutes. [b] Odour types and descriptions of pure compounds found in http://www.thegoodscentscompany.com (188). [c] Odorant descriptors found in a comprehensive literature search (31, 60-62, 64, 68, 121, 122, 186, 187). n.f., not found.

4.3.1.2.Partial least squares-discriminant analysis (PLS-DA)

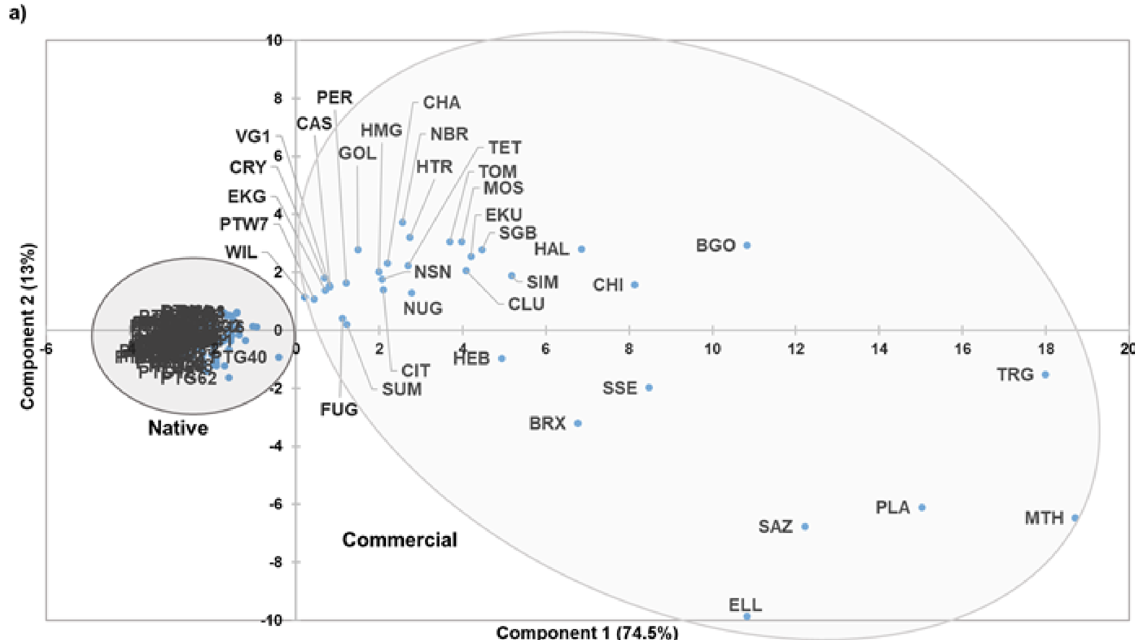
Even though the heatmap is a great visual tool for a preliminary investigation of the data set, it does not provide a comprehensive statistical evaluation of the contribution of each compound to sample differentiation. For that reason, partial least squares-discriminant analysis (PLS-DA) was conducted to discriminate the commercial and Portuguese hops based on the 32 volatile compounds. PLS-DA is a well-known supervised classification method popular that integrates the characteristics of partial least square regression with the discrimination strength of classification methods (189). This technique is based on linking two data matrices, X (explanatory dataset) and Y (explicative dataset) and aims to maximize the covariance between both datasets. The main advantage of the PLS-DA approach is its ability of handle highly collinear and noisy data (190).

From the 32 volatile compounds selected, β-myrcene was not included for PLS-DA, as it did not present significant differences between commercial and Portuguese hop samples and therefore, would not contribute to distinguish them. In order to obtain a successful regression model, R^2X and R^2Y had to be equal or superior to 0.700. The Receiver Operator

Characteristic (ROC) was the statistic evaluated for the ability to predict class membership (191). This statistic is a combination of sensitivity (how well the model is able to correctly classify samples of the class of cases) and specificity (how well the model can predict samples from the class of controls) (192). The curve resulting from plotting the sensitivity against 1-specificity provides the area under the ROC (AUROC), which should be superior to 0.500 in order to discriminate between classes. The PLS-DA model was successfully obtained using five principal components ($R^2X = 0.714$ and $R^2Y = 0.961$). Regarding the ability to predict class membership, a perfect discrimination between classes was obtained (AUROC = 1.000) for both prediction and validation.

The scores resulting from the PLS-DA model were combined in a bi-plot (Figure 4.4a) and the observed first two components accounted for 89.8% of the total variance among samples. In PLS-DA score plot, commercial and Portuguese hops (except for PTW7) samples were separated along component 1, indicating significant differences in volatile compounds profiles of the two groups.

Values of variable importance in projection (VIP) were calculated for the selected compounds to identify which volatile compounds could explain the separation of Portuguese native and commercial hops. In the PLS-DA discriminant process, the independent variables are usually considered to have important role when VIP values are higher than 1.0 (193, 194). A total of 12 volatile compounds with VIP > 1.0 were identified (Figure 4.4b), indicating that these compounds were the main responsible for the separation of the two groups as shown in PLS-DA score plot (Figure 4.4a).



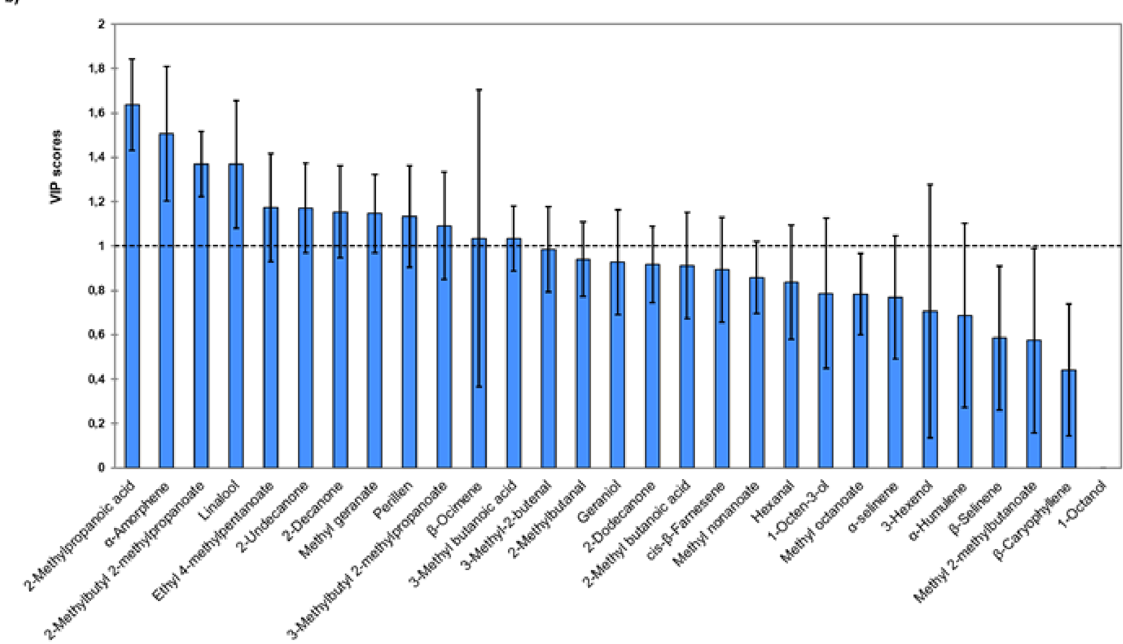


Figure 4.4. Multivariate analysis of volatile compounds using PLS-DA between commercial and Portuguese hop samples. **a)** PLS-DA score plot. **b)** Volatile compounds ranked by VIP scores Variable importance for the projection (VIP) obtained from PLS-DA analysis with 95% confidence interval. Highly influential latent variables have VIP > 1.

Other authors also demonstrated that the different composition in volatile profile can be used to discriminate and identify hops varieties. In studies with twelve cultivars (Styrie,

SAZ, LUB, MTH, HAL, NBR, NUG, POR, Northdown, Galena, TRG, and CHA) fourteen compounds were selected for hop discrimination, including 7 terpenic compounds (bergamotene, β-farnesene, α-humulene, α-amorphene, α- and β-selinenes and humulene epoxyde II), 6 esters (3-methylbutyl 2-methylpropanoate, 2-methylbutyl 2-methylpropanoate, methyl-4-decenoate, methyl geranate and methyl 3,6-dodecanoate) and one methyl ketone (2-undecanone) (195, 196). Jorge and Trugo (2003) used five markers (myrcene, α-limonene, β-caryophyllene, aloaromadendrene and linalool) normalized relatively to α-humulene to discriminate 18 varieties of hops (112). High levels of α- and β-selinenes, methyl geranate and geraniol were determinat in the differentiation of TOM cultivar in comparison with the bitter varieties NSN and NUG, and the aromatic CAS and SAZ (186).

Results pointed that 2-methylpropanoic acid was the main compound to distinguish the tested hops, followed by α-amorphene, 2-methylbutyl 2-methylpropanoate, linalool, ethyl 4-methylpentanoate, 2-undecanone, 2-decanone, methyl geranate, perillen, 3-methylbutyl 2-methylpropanoate, β-ocimene and 3-methylbutanoic acid (Figure 4.4b). This was, in part, in agreement with a recent study with three German hops that suggest myrcene, linalool, and 2- and 3-methylbutanoic acid are important hop odorants present in different concentration among the varieties (121).

However, it is important to take into consideration that, besides differences among hop varieties, the quantity of some compounds can also be influenced by environmental factors such as the growing area, virus infection, and cultivar age (197). It is demonstrated for example, that the Cascade hops grown in United States have higher linalool contents than the ones grown in Hallertau region of Germany. On the other side, Germany crops present higher quantities of the esters 2-methylpropyl- and 2-methylbutyl-2-methylpropanoate (198).

4.3.2. Sensory analysis

CATA questions are a simple method to gather information about panel perception of the sensory characteristics of food products (199). Cochran's Q test is then used to determine if panelists detected significant differences between samples for each of the terms of the CATA question (200). Correspondence analysis is then used on a matrix containing the number of consumers who checked each term from the CATA question to describe each sample, in order to obtain a sensory map of the samples. This analysis allows the determination of similarities and differences between the samples, as well as the sensory attributes that characterize them (200).

Results from an overall evaluation of the 109 hop samples are presented in the Supplementary Table 4 that shows Cochran's Q test values as well as the proportions of selection by the semi-trained panel across all samples for individual aroma attributes listed on the CATA question. Although the semi-trained panel detected significant differences ($p < 0.050$) among the 109 hop samples for every aroma attribute, it was not possible to observe a distinguishable profile for each one. The same tendency was detected when analysing the results obtained from correspondence analysis (Figure 4.5), even though it is very likely that real differences exist between the hop samples in terms of their aroma profiles ($p < 0.001$, with 73.86% of total inertia on the first two dimensions).

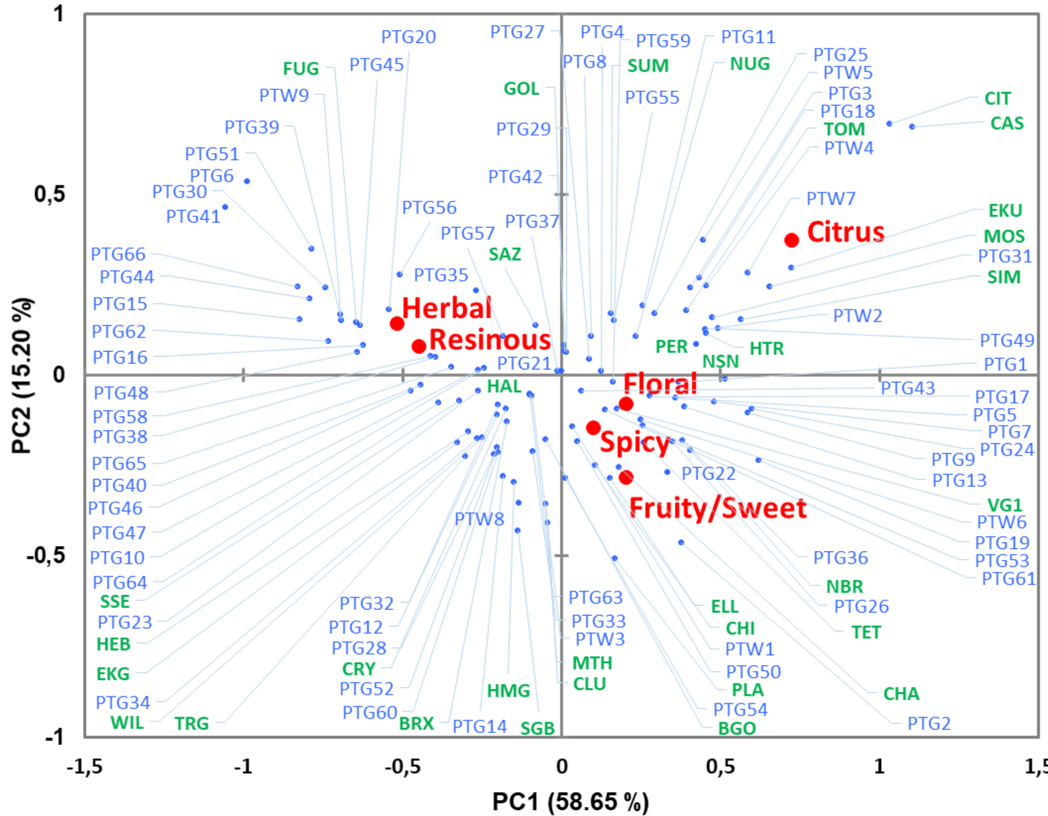


Figure 4.5. Distribution of hop samples in the different aroma regions (red) considering the 34 commercial (green) and 75 Portuguese native hops (blue).

Regarding the overall evaluation of commercial and Portuguese native hop samples, the results obtained from CATA analysis are presented in Table 4.3. The semi-trained panel was able to distinguish them ($p < 0.050$) for 3 attributes: citrus, fruity/ sweet, and floral aroma.

The proportions of selection of those attributes were higher (i.e., more frequently selected by the semi-trained panel) for commercial hop samples. Results obtained from correspondence analysis (Figure 4.6) showed that the aroma profile of commercial and Portuguese hops are likely different (p = 0.002, with 100.00% of total inertia on the first two dimensions). The aroma profile of commercial hops was related to citrus, fruity/ sweet and floral aroma (in agreement with what was observed from the contingency table), whereas Portuguese hops were related to resinous, spicy, and herbal.

Table 4.3. A contingency table of the proportions of selection by 16 semi-trained panellists across Commercial and Portuguese hop samples for individual terms of the Check-All-That-Apply (CATA) question.

Attributes	Commercial	Portuguese	*p*
Citrus	0.293 b	0.017 a	0.000
Fruity/Sweet	0.448 b	0.172 a	0.002
Floral	0.345 b	0.086 a	0.002
Spicy	0.241	0.241 a	1.000
Resinous	0.328	0.293	0.670
Herbal	0.276	0.310	0.683

Cochran's Q test was performed to determine whether the proportions of selection by the semi-trained panel for individual terms of the CATA question differed as a function of hop type. Post-hoc multiple pairwise comparisons were performed using Marascuilo's test. The proportions with different letters within each row represent a significant difference at $p < 0.050$.

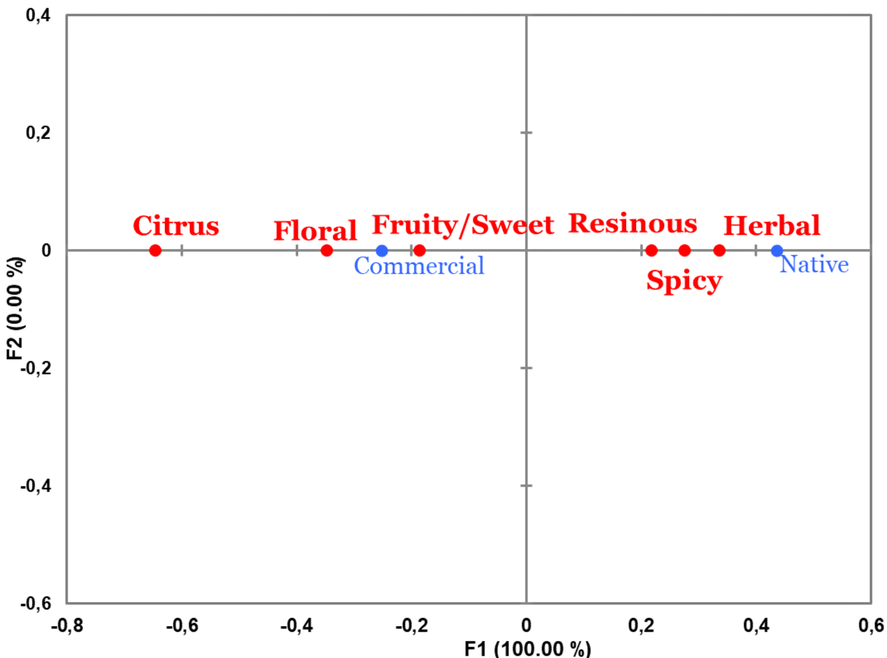


Figure 4.6. Distribution of hop samples in the different aroma regions (red) considering the 109 hop samples grouped by commercial and Portuguese native hops (blue).

4.3.3. Correlation of sensory characteristics with volatile profile

PLS regression was performed in order to study the correlation of aroma data with analytical data (volatile profile), based on aroma data prediction (Y-variables) from analytical data (X-variables). For this analysis, only aroma attributes and volatile compounds with significant differences between commercial and Portuguese hops were considered. Table 4.4 summarizes individual aroma attributes prediction from analytical parameters; the importance of volatile profile in the projection and their standardized coefficients was also determined, and latent variables were identified. An effective regression model, with good predictive ability could indicate an influence of volatile profile on aroma profile, regardless the hops. All the 3 aroma descriptors analyzed were found to be correlated with volatile compounds. Regression models were considered successful when $R^2 \geq 0.70$, and presenting good ability to predict new samples when $Q^2 \geq 0.50$ (201). Good regression models, with good predictive ability were found for all aroma descriptors (citrus, fruity/sweet, and floral).

Table 4.4. Results of PLS regression between hops volatile composition (X-variables) and hops sensory aromas (Y-variables).

Sensory aroma	Q^2	R^2Y	R^2X	RSME	Latent variables [a]
Citrus	0.807	1.019	0.978	0.034	**Ethyl 4-methylpentanoate; 2-Undecanone (-); 2-Decanone; Methyl geranate; Linalool;** 3-Methyl butanoic acid; Perillen; β-Ocimene; 2-Methylpropanoic acid.
Fruity/Sweet	0.604	0.989	0.997	0.075	**3-Methylbutyl 2-methylpropanoate; 2-Undecanone (+); 2-Methylbutyl 2-methylpropanoate; Ethyl 4-methylpentanoate; Methyl geranate;** α-Amorphene; Linalool; Perillen; 2-Decanone; 3-Methyl butanoic acid.
Floral	0.923	1.017	0.997	0.029	**Linalool (-); 3-Methyl butanoic acid (-); 2-Methylpropanoic acid; Methyl geranate; 2-Undecanone; 2-Decanone; Perillen; β-Ocimene.**

[a] Latent variables with significant weight in the model and correlation with Y-variable; highly influential latent variables (variable importance for the projection>1) are represented in bold and the remaining are moderately influential latent variables (0.8<variable importance for the projection<1). (+), significant positive correlation with Y-variable; (-), significant negative correlation with Y-variable. Q^2, cumulative predictive variation from internal cross-validation; R^2X, cumulative explained variation of X explained in terms of sum of squares; R^2Y, cumulative explained variation of Y explained in terms of sum of squares; RMSE, Root mean square error.

Regarding citrus aroma, ethyl 4-methylpentanoate (described by olfactometry in hops as sweet, fruity, citrus, pineapple), 2-undecanone (floral, citrus), 2-decanone (earthy, moldy, musty), methyl geranate (greenery) and linalool (sweet, flowery, citrus-like, terpenic, fresh) were highly influential latent variables (variable importance for the projection, VIP, > 1), with 2-undecanone showing a negative influence on citrus perception. Fruity/ sweet aroma attribute was highly influenced by the percentage of 3-methylbutyl 2-methylpropanoate (fruity, spicy, sweet), 2-undecanone (floral, citrus), 2-methylbutyl 2-methylpropanoate (cooked vegetable), ethyl 4-methylpentanoate (sweet, fruity, citrus, pineapple), methyl geranate (greenery), and, contrary to citrus, the fruity/ sweet sensation was higher when 2-undecanone was present in higher percentage. These results could suggest that the panel perception of citrus and fruity/sweet were related to the percentage of 2-undecanone and were influenced in opposite directions (fruity/sweet with higher percentage and citrus with lower). Although 2-undecanone has been described as citrus and floral in olfactometry of

hops (62), the profile observed in this study resembles the fruity notes description of pure compound (202, 203).

As for floral aroma, linalool (sweet, flowery, citrus-like, terpenic, fresh), 3-methyl butanoic acid (goaty, sweaty, cheesy), 2-methylpropanoic acid (sweet, malty), methyl geranate (greenery), 2-undecanone (floral, citrus), 2-decanone (earthy, moldy, musty), perillen (citrus), β-ocimene (green, floral) were the compounds with high influence on this attribute. Also, 3-methyl butanoic acid and β-ocimene had a negative influence on floral sensation.

4.3.4. Conclusion

This study contributed to the characterization ofvolatile profile of Portuguese native hops and can be useful to develop new varieties for the modern beer trends. Chemometric techniques were found relevant tools to study volatile profile and aroma attributes and understand relationships between them.

Overall, Portuguese native hops had different volatile profiles and aroma properties in comparison with commercial hops selected from the most representative market varieties. PSL-DA model identified 12 volatile compounds responsible for the separation of commercial and Portuguese hops. Two Portuguese samples coded as PTW7 and PTG40 stood out from the others as they were consistently separated from the other Portuguese native hops. Moreover, PTW7 appeared to be similar to reputable varieties of worldwide market, as NBR, NUG and CIT. In general, Portuguese hops were related to resinous, spicy, and herbal aroma characteristics, whilst commercial hops were more citrus, fruity/ sweet, and floral.

PLS regression models provided information on the relationship between aroma characteristics and volatile profile, regardless of hop variety. Successful models were obtained for citrus, fruity/ sweet, and floral. Nevertheless, these relationships should be interpreted as associations and not as direct cause and effect, once observed correlations do not necessarily imply causality.

PART III

Impact of hops in beer sensory attributes and composition

CHAPTER 5

Portuguese native hops: impact on sensory and analytical profile of dry-hopped beers

5.1. Introduction

Beer organoleptic quality in what concerns hop-derived flavours is primarily based on the chemical characteristics of the cone. However, the solubility of aroma compounds into the beer liquid is also important to examine the differences between hop samples alone and hop samples in beer (13). Dry-hopping is becoming increasingly popular to impart intense hoppy flavour to beers, covering a great variety of sensory impressions from floral, fruity, green-grassy to herbal, spicy, woody, and many more (13, 204). Traditionally, the dry-hopping technique was used to improve the microbiological stability of beer, but several studies are focused on dry-hopping techniques to evaluate sensory and analytical profiles of hops and beers (14, 16, 19, 68, 205, 206). The quantification of key flavour compounds is important to achieve a better knowledge of the relationship between flavour impression of beer and odour-active compounds of hops (205). However, the sensory perception of beer results from a high number of factors and predicting the aroma and flavour of beer is very complex due to synergistic, antagonistic, and masking effects that occur (13, 204). Human assessors are thus needed to search for potential wild hops that present interesting brewing characteristics. Sensory analysis and gas chromatography-olfactometry (GC-O) are valuable tools for the evaluation of organoleptic characteristics and odour-active compounds of hops and beers (61, 124).

The aim of this study was to investigate the sensory impression and odour-active volatile composition of hops and dry-hopped beers from selected Portuguese wild hop genotypes.

5.2. Materials and methods

5.2.1. Chemical reagents

Reference standards (+)-β-Pinene (≥ 98.5%), 1-Octen-3-ol, borneol (≥ 95%), butyric acid (≥ 99.5%), citral (≥ 95%), *cis*-3-hexen-1-ol (≥ 98%), diacetyl, dimethyl sulphide, dimethyl trisulphide (≥ 98.5%), 3-methylbutanal (≥ 97%), 2-methylbutanal (≥ 95%), ethyl 2-methylbutanoate (99%), ethyl 2-methylpentanoate (internal standard, ≥ 99%), ethyl 2-methylpropanoate (≥ 99%), ethyl 3-methylbutanoate (≥ 98%), ethyl 4-methylpentanoate (≥ 97%), ethyl butanoate (≥ 99.5%), ethyl hexanoate (≥ 99.5%), eugenol (99.6%), furaneol (≥ 99%), geraniol (≥ 99%), hexanol (≥ 99%), 3-methylbutyl acetate (≥ 97%), linalool (97%), menthol (99%), methyl nonanoate (≥ 99.8%), myrcene (≥ 90%), dimethyl disulphide (≥

90%), limonene (97%), hexanal (≥ 98%), 3-hexenol (≥ 95%), 2-phenyl ethanol (≥ 99%), β-caryophyllene (≥ 80%), α-humulene (≥ 96%), humulene oxide, S-methyl hexanthioate, S-methyl 4-methylpentanoate and theaspirane (≥ 90%) were purchased from Sigma Aldrich (St. Louis, Mo., US).

5.2.2. Steam distillation

The distillation apparatus included a boiling flask (2 L), a condenser and a calibrated distillation receiver, graduated in 0.1 mL intervals. Grounded hops (from 25 to 50 g) and water were placed in boiling flask, in the proportion of 60 g per liter. The system was brought to boiling in a heating mantle for 4h, in a approximated rate of 8 mL/ min (113, 114).

5.2.3. Dry hopping trials

Commercial beer (Munich-Style Helles) was the base beer for dry-hopping experiments. Dry-hopped beers were prepared in 10 L kegs (Cornelius Deutschland GmbH, Langenfeld, Germany). After hops addition (3 g/ L), kegs were closed and filled with CO_2 (1.5 bar) to carry the procedure free of oxygen. Beer (8 L) were added and kegs kept in agitation per 6 days at 4 ºC. Non dry-hopped beer was carried in the same conditions without addition of hops. All trials were done in triplicate. Hop concentration and maturation time were determined taking in consideration previous studies of extraction of volatile compounds in dry-hopping techniques and the practical uses of breweries (19, 36, 68). Hop genotypes to be used in the present assay were selected from previously characterized samples accordingly to the genetic diversity (Chapter 2) and chemical parameters (Chapter 3). Additionally, total oil content herein determined was also included as a selection criterion. However, this study included the harvest year of 2017.

5.2.4. Sensory evaluation

Sensory assays were performed individually in standard cabins and samples were blind-labelled with a three-digit code. Hop samples were presented in original stored vacuum bags, whereas beers analysis was performed in fresh samples (after 6 days of maturation time). All assessors (20–50 years of age) were trained for evaluation of beer and certified by the German agricultural society (Deutsche Landwirtschafts-Gesellschaft e.V.). Those asserssors also performed the evaluation of raw material sensory characteristics.

Check-All-That-Apply test (CATA) was performed to evaluate ortho-nasal hop odour. Flavour profile of beer samples was done by Quantitative Descriptive Analyses (QDA), providing a complete sensory description, taking into account the global sensation perceived (olfactory and gustatory). Each assessor rated the descriptors intensity on a five-point scale (0 = imperceptible, 1 = very weak, 2 = weak, 3 = middle, 4 = intensive, and 5 = very intensive), the averaged results for each beer were plotted in a radar diagram. The sensory evaluations of beer samples were performed in triplicate at room temperature (20 ± 1 °C).

Citrus, green fruits, sweet fruits, floral, woody, green grassy, earthy, resinous, green tea, spicy, herbal, and sulphurous percepection were analysed in hops and beers. Total hoppy impression was also evaluated in beers samples. During the sessions, standard references and known commercial varieties of hops (Table 5.1) were avaible to panellists, to best define hops and hops-derived attributes.

Table 5.1. Attributes, standard and hop references provided to panelists during training sessions.

Attributes	Standard references
FRUITY	
Citrus (lemon, orange, tangerine)	Citral 30 µg/ L
Green fruits (pear, apple)	Hexanol 70 µg/ L
Red berries	Ethyl 3-methylbutanoate 40 µg/ L
Tropical fruits (pineapple, strawberry)	Ethyl hexanoate 20µg/ L
Sweet fruit (banana, ice bonbon)	Isoamyl acetate 1.1 mg/ L
VEGETAL	
Floral	Linalool 7 µg/ L
Woody	Borneol 6 µg/ L
Menthol	Menthol 600 mg/ L
Green grassy	*cis*-3-hexen-1-ol 0.25 µg/ L
Mushroom	1-Octen-3-ol 100 µg/ L
Resinous (pine, cedar)	(+)-β-Pinene 80 µg/ L
Green tea	Theaspirane 4 µg/ L
Rose, floral, honey	2-Phenyl ethanol 10 mg/ L
SPICE	
Spicy (curry, cloves)	Eugenol 130 µg/ L
Herbal and spicy	Myrcene 0.1 mg/ L
OTHERS	
Cheese	Butyric acid 240 µg/ L
Cream caramel (sweet-like)	Furaneol 4 µg/ L
Cooked vegetable	Dimethyl sulphide 0.1 mg/ L
Butter	Diacetyl 6.5 µg/ L
Sulphurous (garlic, onion, leek)	Dimethyl trisulphide 25 µg/ L
Sweaty and cheese	Ethyl butanoate 240 µg/ L

Hops	Attributes and descriptions[1]
ANA	**Fruity**: black berries, blackcurrant, peach, pear, tropical fruits, resinous, grapefruit, strawberry, quince, green pepper, banana
BRO	**Citrus and herbal**: orange, fruity, vanilla, floral, chrysanthemum, vanilla cream, vegetable, calendula, butter
NUG	**Fruity, spicy and resinous**: pineapple, lemon, ginger, geranium, floral, lychee
HBC	**Fruity and spicy**: white wine, coffee, cassis, gooseberry, grapefruit, lemon grass, elderflower, grapes
HMG	**Spicy and green fruits**: fruity, apple, pepper, lemon, chocolate, green peppers, mint
HTU	**Spicy and fruity**: pepper, lime, currant, spicy, plain chocolate, ripe banana, pepper, curry
HTR	**Herbal and citrus**: tea, spicy, orange, lavender, cassis, apricot, citrus, peach
HKS	**Citrus, fruity and spicy**: pepper, spicy, resinous, orange, honeydew melon, lemon, melissa
HEB	**Herbal and green tea**: spicy, hay, orange, tobacco, citrus, black tea, marjoram, ginger, melissa
HMN	**Fruity and sweet**: melon, tropical fruit, orange, vanilla, fruit tea, wild strawberry, geranium, aniseed
MBA	**Citrus and fruity**: tangerine, grapefruit, lime, bubble gum, pineapple, gooseberry, cassis, strawberry, lemon
OPL	**Spicy**: herbal, pepper, grass, aniseed, citrus, apricot, liquorice, aniseed, bergamot
PLA	**Citrus and fruity**: menthol, ice wine, pineapple, pineapple, woodruff, bergamot, banana, mint
SGD	**Herbal, spicy and resinous**: spicy, aniseed, tobacco, clove, cognac, camomile tea, liquorice, tarragon, butter

[1] Adapted from Hopsteiner and Barth-Haas Group. Reference hops, ANA, BRO, HBC, HMG, HTU, HTR, HKS, HMN, MBA, OPL, PLA, SGD, all harvest 2017, were provided from the Gesellschaft fuer Hopfenforschung e.V. (Wolnzach, Germany).

5.2.5. Gas-chromatography for olfactometry of hops and quantification of beer compounds

5.2.5.1. Extraction of volatile compounds by Headspace solid phase micro-extraction (HS-SPME)

Hop volatile compounds were extracted by headspace-solid phase micro-extraction: 0.5 g of hops were placed in 20 ml headspace vials with polypropylene caps (Butyl/ PTFE, Achroma, Mühlheim, Germany) exposed to a divinylbenzene/ carboxen/ polydimethylsiloxane (DVB/ CAR/ PDMS) SPME fiber 50/30 µm (Supelco/Sigma Aldrich, Bellafonte, Penn.,US) for 30 min at 40 °C and analysed by gas chromatography-mass spectrometry-olfactometry (GC-MS/O) (68). For quantification of selected volatile compounds in beer, 5.0 g were placed in 20 ml headspace vials and extracted by headspace-solid phase micro-extraction using a similar procedure.

5.2.5.2. Gas-chromatography mass-spectrometry/ olfactometry (GC-MS/O) parameters

Chromatographic analysis was performed in the gas chromatograph system TRACE 1300 Ultra directly coupled with an ISQ QD single quadrupole mass spectrometer (ThermoScientific, Waltham, Mass., US) equipped with an injection port split/ splitless associated with a selective detector mass, EI mode at an ionization energy of 70 eV. The GC-MS was equipped with a Trace GOLD TG-5MS (ThermoScientific Waltham, Mass., US) column (60 m X 0.25 mm X 0.25 mm). After volatiles extraction HS-SPME fiber was desorbed at injection port at 250 °C for 0.5 min in splitless mode. For the chromatographic separation, the GC oven temperature starts at 60 °C, held for 4 min, increased at 5 °C per minute to 220 °C, held for 5 min and heated to 250 °C at a rate of 10 °C per minute and the final temperature held for 2 min. The transfer line was set to a temperature of 250 °C. The mass spectrometer detected mass ranges between 35 and 350 u. The chromatographic separation had a constant flow of 1.2 ml/ min using as carrier gas helium BIP. The retention index (RI) of each compound was calculated using the retention time (RT) of that compound compared against the RTs of a series of standard n-alkanes. The compounds were identified based on their retention indices, odour perceptions and the mass spectra of NIST 11 library or authentic standards measured under the same measuring conditions.

Odour-active volatile compounds of hops were identified by olfactometry. A trained GC-MS-O analyst was asked to describe the perceived odours as well as their intensity. Method of Odour Intensity was used with a 4-point scale (not detected, weak, moderate, and strong).

The quantification of selected volatiles in beer samples was done using an internal standard (IS, ethyl 2-methylpentanoate 0.02 μg/ mL). Three esters (ethyl 2-methylpropanoate, ethyl 2-methylbutanoate, ethyl 4-methylpentanoate), three terpenes (myrcene, linalool and geraniol) and two sulphur compounds (dimethyl trisulphide (DMTS) and S-methyl hexanthioate) were selected as relevant odour-active compounds of the three major groups found in Portuguese hops. Besides, three sesquiterpenes (β-caryophyllene, α-humulene and humulene oxide) usually found in hops were also quantified, although they were not detected in GC-O trails. Calibration curves at six concentration points made on beer matrix (Munich-Style Helles).

5.2.6. Statistical analysis

All dependent variables analysed were tested for distribution of the residuals with Shapiro–Wilk's test. Regarding CATA tests applied to hop samples, Cochran's Q test (182) was performed to determine whether the proportions of selection by the semi-trained panel for individual attributes of the CATA question differed as a function of hop sample. If there was a significant difference among the variables, post hoc multiple pairwise comparisons were performed using Marascuilo's test. Concerning olfactometry of hops, Principal Component Analysis (PCA) was used to summarize odour descriptors and intensity of hop samples. For each hop, the sums of odour intensities for each descriptor were calculated taking in consideration the odour intensities of all compounds that showed the same descriptor.

Regarding beer flavour profile, comparison of sensory QDA scores between no dry-hopped and dry-hopped beers was carried out by t-student or Maan-Whitney test depending on normal distribution of the residuals was confirmed or not, respectively. Results from the quantification of beer volatile compounds were analysed by ANOVA and Tukey's post hoc test, since normal distribution of residues and homogeneity of variance was confirmed. Statistical analyses were performed at 5% significance level, using XLSTAT® for Windows versions 2016.02 (Addinsoft, Paris, France).

5.3. Results and discussion

5.3.1. Selection of native hops for beer production

Native hops were previously characterized concerning α-acids, β-acids and xanthohumol (Table 3.2) and genetic relatedness (see Figure 2.1). Notwithstanding total oil content is an important parameter for hops characterization, the steam distillation method requires a relatively large amount of sample, which for same native hops was not possible, thus, total oil was evaluated only in 44 samples. The average content was 0.71 ± 0.36 mL/ 100g, ranging from 0.23 to 1.84 mL/ 100g (Table 5.2).

Table 5.2. Total oil of Portuguese native hops.

Sample	mL/ 100g	Sample	mL/ 100g	Sample	mL/ 100g
PTG22	0.66	PTG38	0.80	PTG57	0.69
PTG23	0.23	PTG39	1.05	PTG58	0.42
PTG24	0.51	PTG42	1.50	PTG59	0.59
PTG25	0.79	PTG43	0.41	PTG60	0.57
PTG26	0.34	PTG44	0.80	PTG61	0.71
PTG27	0.53	PTG45	1.09	PTG62	0.68
PTG28	0.53	PTG47	1.84	PTG63	1.32
PTG29	0.77	PTG48	0.00	PTG64	0.51
PTG30	0.62	PTG49	0.53	PTG65	0.63
PTG31	0.74	PTG50	0.46	PTW2	0.68
PTG32	0.61	PTG51	1.79	PTW3	0.59
PTG34	0.60	PTG53	0.38	PTW4	0.25
PTG35	0.67	PTG54	0.86	PTW7	0.39
PTG36	0.92	PTG55	0.87	PTW8	0.47
PTG37	0.84	PTG56	0.81		

PCA was performed using as variables data from α-acids, β-acids, xanthohumol (Table 3.2) and total oil content (Figure 5.1). It is possible to observe that, 3 samples, namely PTW2, PTW7 and PTW8, appeared separated from most of the hops, presenting a compromise between higher content of the four quantified variables. These hops were selected for beer production. In addition, taking into consideration the technical limitations concerning the maximum number of hops to be used in the beers production, PTG22 was selected to represent native hops that have low amount of all variables but is genetically similar to other selected hop (PTW8).

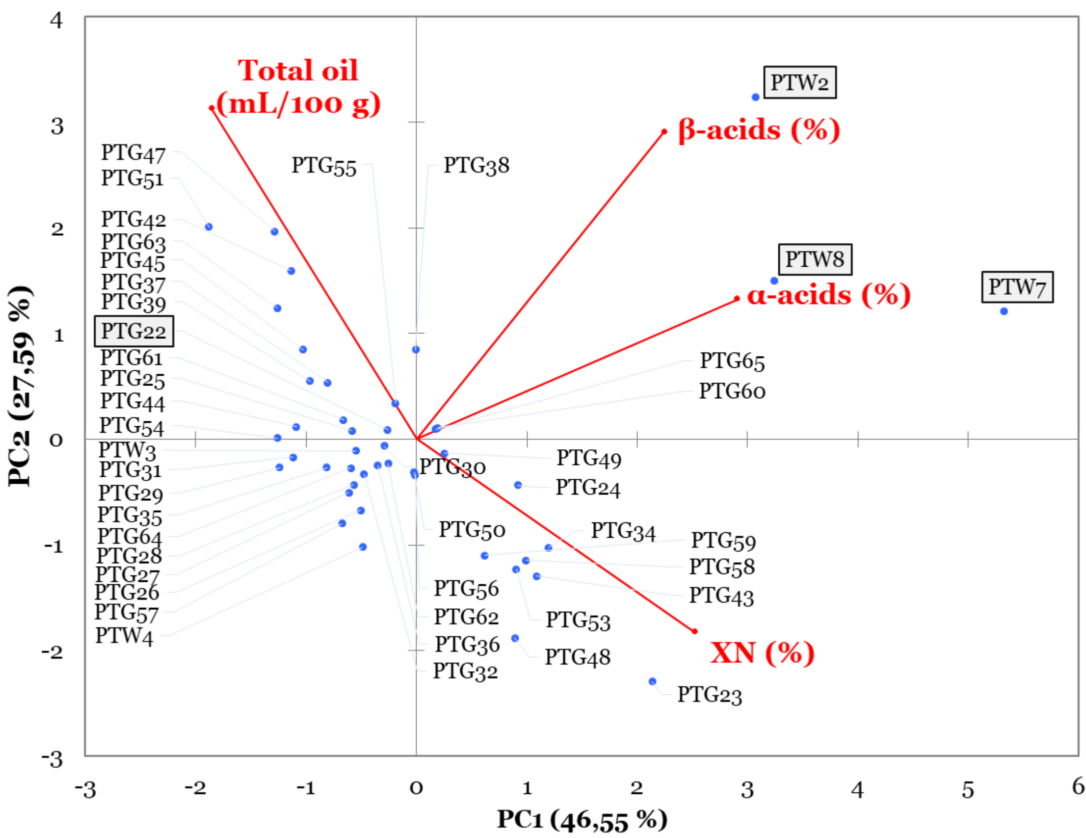


Figure 5.1. Principal Component Analysis (PCA) of the relation of Portuguese native hops and α-acids, β-acids, xanthohumol, and total oil quantification.

5.3.2. Sensory analysis of selected hops

From CATA analysis (Table 5.3), Cochran's Q test was applied to each attribute for identification of relevant sensory characteristic of hops. Significant differences were found in fruity (including citrus, green fruit, and sweet fruit), floral and woody attributes. Contrasting results were found for PTW7 and PTG22, being PTW7 the fruitiest and floral, and PTG22 the woodiest sample. PTW2 showed less citrus and green fruity characteristics than PTW7. PTW8 presented less green fruity and woody impression than PTW7 and PTG22 respectively.

Table 5.3. Sensory analysis of Portuguese hops performed by Check-All-That-Apply (CATA) test.

	PTW2	PTW7	PTW8	PTG22	*p*-values
Citrus	25 [a]	82 [b]	45 [ab]	25 [a]	0,001
Green fruits	45 [a]	77 [b]	36 [a]	35 [a]	0,003
Sweet fruits	50 [ab]	86 [b]	64 [ab]	20 [a]	0,004
Resinous	50	45	50	45	0,463
Woody	40 [ab]	18 [a]	18 [a]	65 [b]	0,022
Earthy	20	0	9	40	0,080
Floral	45 [ab]	77 [b]	50 [ab]	25 [a]	0,021
Green grassy	30	45	23	15	0,364
Green tea	35	27	14	45	0,245
Spicy	75	68	59	75	0,708
Herbal	50	23	41	50	0,599
Sulphurous	15	23	41	30	0,137

Values are in % of times that the attribute was identified by the panellists. Cochran's Q test was performed to determine whether the attributes differed as a function of hop sample. Different letters in the same row, represent significant differences ($p < 0.05$) for each descriptor by Marascuilo's test.

The high fruity and floral impression observed on PTW7, was expected since this sample is genetically close to the Nugget variety that presents similar sensory characteristics.

No significant statistical differences among the samples were observed for resinous, earthy, green grassy, spicy, herbal, and sulphurous attributes.

5.3.3. Olfactometry of selected hops

Gas chromatography coupled with mass detection and olfactometry of the four selected hop samples allowed to identify 38 odour-active compounds and quantify their odour intensity (Table 5.4).

Table 5.4. GC–O Identified Aroma-Active Compounds in wild hops with the Method of Aroma Intensity.

RI[a]	Compound	Id	Compound class	Odour descriptors	OI
400	Methyl mercaptan	RI, MS, OD	Thiol	Sulphurous	2;2;1;1
545	Diacetyl	RI, Std, OD	Ketone	Rancid	0;1;0;0
644	3-Methylbutanal	RI, Std, OD	Aldehyde	Fruity, green fruit	0;1;1;0
652	2-Methylbutanal	RI, Std, OD	Aldehyde	Fruity, green fruit	0;2;1;0
678	Methyl 2-methylpropanoate	RI, MS,OD	Ester	Fruity, tropical fruit	0;1;0;0
741	2-Methylpropanoic acid	RI, MS,OD	Carboxylic acid	Fruity, rancid, cheese	0;1;0;0
747	Dimethyl disulphide	RI, Std,OD	Thioether	Sulphurous, garlic, onion	0;1;0;1
753	Ethyl 2-methylpropanoate	RI, Std,OD	Ester	Fruity, sweet fruit	1;1;1;1
774	Methyl 2-methylbutanoate	RI, MS,OD	Ester	Fruity, green fruit	2;2;1;2
798	S-methyl propanthioate	RI, MS,OD	Thioester	Sulphurous	1;0;0;0
801	Hexanal	RI, Std,OD	Aldehyde	Green grassy	0;1;0;0
808	$C_2H_4S_2$	RI, MS,OD	Thioether	Sulphurous	3;3;3;3
831	3-Methylbutanoic acid	RI, MS,OD	Carboxylic acid	Rancid, cheese	1;3;1;3
843	2-Methylbutanoic acid	RI, MS,OD	Carboxylic acid	Fruity, rancid, cheese	1;1;1;1
848	Ethyl-2-methylbutanoate	RI, Std,OD	Ester	Fruity, tropical fruit	1;1;1;0
868	3-Hexenol	RI, Std,OD	Higher alcohol	Green grassy	1;1;1;1
941	S-methyl 2-methylbutanthioate	RI, MS,OD	Thioester	Sulphurous, fruity Sweet, tropical, and green fruit	1;1;1;1
964	Ethyl 4-methylpentanoate	RI, Std,OD	Ester	Fruity, sweet fruit, tropical fruit	1;2;1;1
977	Dimethyl trisulphide	RI, Std,OD	Thioether	Sulphurous, garlic, onion	1;0;1;1
980	1-Octen-3-ol	RI, Std,OD	Higher alcohol	Mushroom, earthy	1;0;1;1
991	Myrcene	RI, Std,OD	Monoterpene	Herbal, resinous, spicy	3;3;1;2
1036	Limonene	RI, Std,OD	Monoterpene	Citrus, fruity	1;1;1;1
1050	Ocimene	RI, MS,OD	Monoterpene	Citrus, fruity, herbal	0;2;0;0
1059	S-methyl 4-methylpentanoate	RI, Std,OD	Thioester	Sulphurous, garlic, onion, cooked vegetable	1;0;1;1

[a] calculated by linear interpolation from the retention times of the compound and adjacent n-alkanes.
[b] Method of identification (Id): RI, retention index; Std, confirmed by authentic standards; MS, mass spectrum library; OD, odour descriptor; OI, Odour intensity (0 - 3) PTW2: PTW7: PTW8:PTG22 hops.

Table 5.4. GC–O Identified Aroma-Active Compounds in wild hops with the Method of Aroma Intensity (continued).

RI[a]	Compound	Id	Compound class	Odour descriptors	OI
1062	Methyl 2-methylheptanoate	RI, MS,OD	Ester	Fruity, tropical fruit	1;1;1;1
1093	S-methyl hexanthioate	RI, Std,OD	Thioester	Sulphurous, garlic, onion, cooked vegetable	0;2;1;2
1099	Linalool	RI, Std,OD	Monoterpenoid alcohol	Floral, citrus, fruity	3;3;1;3
1135	Trithio-2,3,5-hexane	RI, MS,OD	Thioether	Sulphurous	3;2;2;1
1168	S-methyl 5-methylhexanthioate	RI, MS,OD	Thioester	Sulphurous, garlic, onion, cooked vegetable	1;1;1;1
1182	Methyl phenylacetate	RI, MS,OD	Ester	Honey	1;1;0;1
1200	S-methyl heptanthioate	RI, MS,OD	Thioester	Sulphurous, fruity, garlic, onion, cooked vegetable	0;0;1;0
1213	S-methylthiomethyl 2-methylpropanthioate	RI, MS,OD	Thioester	Sulphurous, garlic, onion	2;1;1;1
1257	Geraniol	RI, Std,OD	Monoterpenoid alcohol	Floral, citrus, fruity	1;2;1;2
1272	3-(4-Methyl-3-pentenyl)-thiophene	RI, MS,OD	Thioterpene	Rubbery	1;1;0;1
1307	S-methylthiomethyl 3-methylbutanthioate	RI, MS,OD	Thioester	Sulphurous, garlic, onion, earthy	1;0;1;0
1309	S-methylthiomethyl 2-methylbutanthioate	RI, MS,OD	Thioester	Sulphurous, garlic, onion, earthy	2;1;3;1
1431	S-methylthiomethyl 4-methylpentanthioate	RI, MS,OD	Thioester	Sulphurous, garlic, onion, earthy	3;0;3;0
1538	Tetrathio-2,4,5,7-octane	RI, MS,OD	Thioether	Sulphurous, garlic, onion, earthy	1;1;1;1

[a] calculated by linear interpolation from the retention times of the compound and adjacent n-alkanes.
[b] Method of identification (Id): RI, retention index; Std, confirmed by authentic standards; MS, mass spectrum library; OD, odour descriptor; OI, Odour intensity (0 - 3) PTW2: PTW7: PTW8:PTG22 hops.

The following twenty odour descriptors were collected for those 38 odour-active compounds: fruity, citrus, green fruit, tropical fruit, sweet fruit, green grassy, floral, honey, mushroom, earthy, herbal, resinous, spicy, cooked vegetable, rubbery, sulphurous, rancid, cheese, garlic, onion. PCA was applied to summarize the results obtained for olfactometry (odour description and intensity) of hop samples. For each hop sample the sums of odour

intensities for each descriptor were used as variables of the PCA analyses. The sums were calculated taking in consideration the odour intensities of all compounds that showed the same descriptor. For example, myrcene was described as presenting herbal, resinous and spicy odours. For PTW2 and PTW7 the odour intensity of those descriptors was 3, whereas in PTW8 and PTG22 it was 1 and 2, respectively (Table 5.4). However, the herbal odour was also found in ocimene with an odour intensity of 2 found only in PTW7, thus, concerning herbal descriptor, the odour intensities of the four samples were 3 for PTW2, 5 for PTW7, 2 for PTW8 and 1 for PTG22. For resinous and spicy odours only myrcene presented these descriptors, thus, for both descriptors the odour intensities were 3 for PTW2, 3 for PTW7, 1 for PTW8 and 2 for PTG22. Figure 5.2 presents the projection of hop samples (blue) and descriptors (red) in the two main PCA axes (components 1 and 2), representing 87.08% of total variance. PTG22 and PTW8 presented lower intensity of fruity, green fruit, tropical fruit, sweet fruit, green grassy, honey, herbal, resinous, spicy, citrus, rancid and cheese odours, whereas PTW2 and PTW7 presented higher intensities of those descriptors. The highest intensities in fruits descriptors including fruity, citrus, sweet fruit, green fruit, and tropical fruity descriptors were observed in PTW7, which was in agreement with the results from CATA sensory analysis. PTW8 presented higher intensities of mushroom, earthy, garlic, onion and sulphurous descriptors, whereas PTG22 presented higher intensity of cooked vegetable descriptor. However, mushroom, garlic, onion were not detected by panellists on CATA sensory analysis, and regarding the sulphurous and earthy notes, the panellists did not reported differences between those attributes on hop samples. It is described that, depending on their concentration, sulphur compounds can provide undesirable hop flavours of cooked vegetable, musty, cabbage and onion-like to beers (1, 2, 64, 207). However, it is known that, in beers, thioesters can also provide exotic and fruity aromas already related in some tropical fruits (208-210). Therefore, in GC-O analysis, some sulphur compounds were described as undesirable odours of garlic, onions, and cooked vegetable, whereas other sulphur compounds were described with pleasant fruity or earthy odours (68).

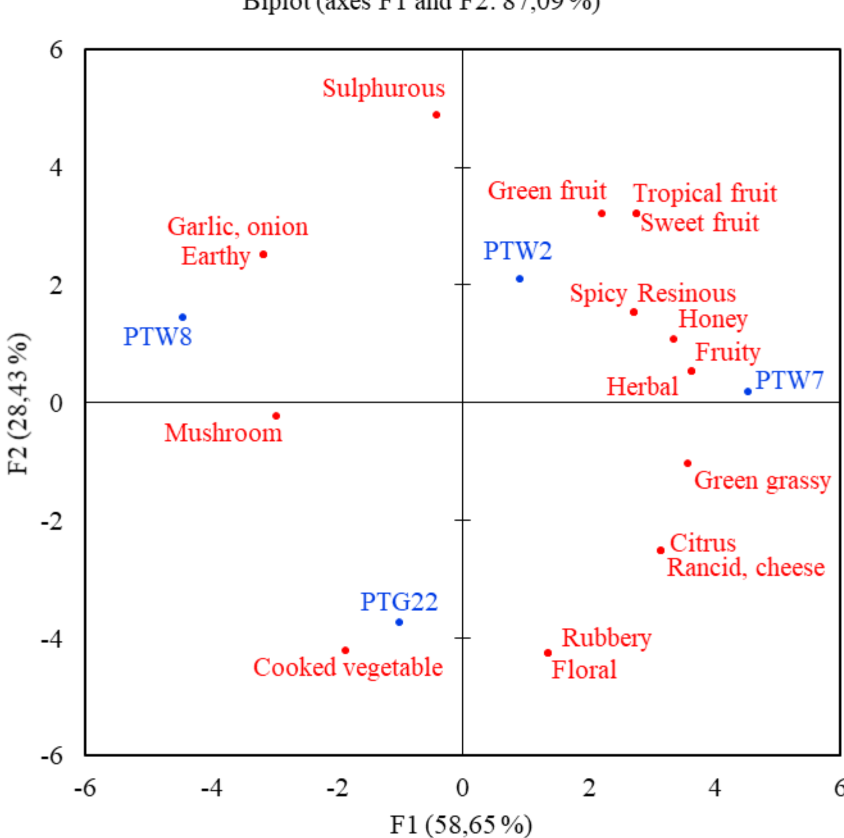


Figure 5.2. Principal Component Analysis biplots of hop samples (blue) and descriptors intensity (red) in the two main axes (components 1 and 2), representing 87.08% of total variance.

In general, the GC-O analysis corroborate the results from CATA sensory analysis and confirmed the fruity pattern of PTW7 and PTW2. The lower intensity of odour active compounds observed for PTG22 hops was also in agreement with CATA sensory analysis. However, woody notes found in CATA test were not identified by GC-O, probably the compounds associated with woody impression, for example sesquiterpenes were below threshold levels (61). However, synergisms can occur that justify the identification of this descriptor in hops.

5.3.4. Sensory analysis of beers

QDA data from dry-hopped beers and base beer (not dry-hopped) are summarised in Figure 5.3. Mann-Whitney test was applied to detect which attributes were significantly different after dry-hopping. As expected, all dry-hopped beers presented higher scores of total hop impression in comparison with base beer (no dry-hopped) (Figure 5.3). Beers dry-

hopped with PTW2 and PTW7 hops presented a significant increase of citrus attribute. PTW2 beer also provided the highest score of sweet fruit sensation, and differences were also detected in spicy characteristics of beers dry-hopped with PTG22 hops. Higher fruity impression was expected for beer dry-hopped with PTW7 due to the its intense fruit odour, however, this beer presented lower fruity impression than PTW2 dry-hopped beer, which was not observed on CATA and GC-O analyses of hops. No unpleasant sulphurous sensation was observed on dry-hopped beers. Hop odour character can change from whole cone hops to dry hopped beer, however, the same odour descriptors remained useful in describing hop and beer odour.

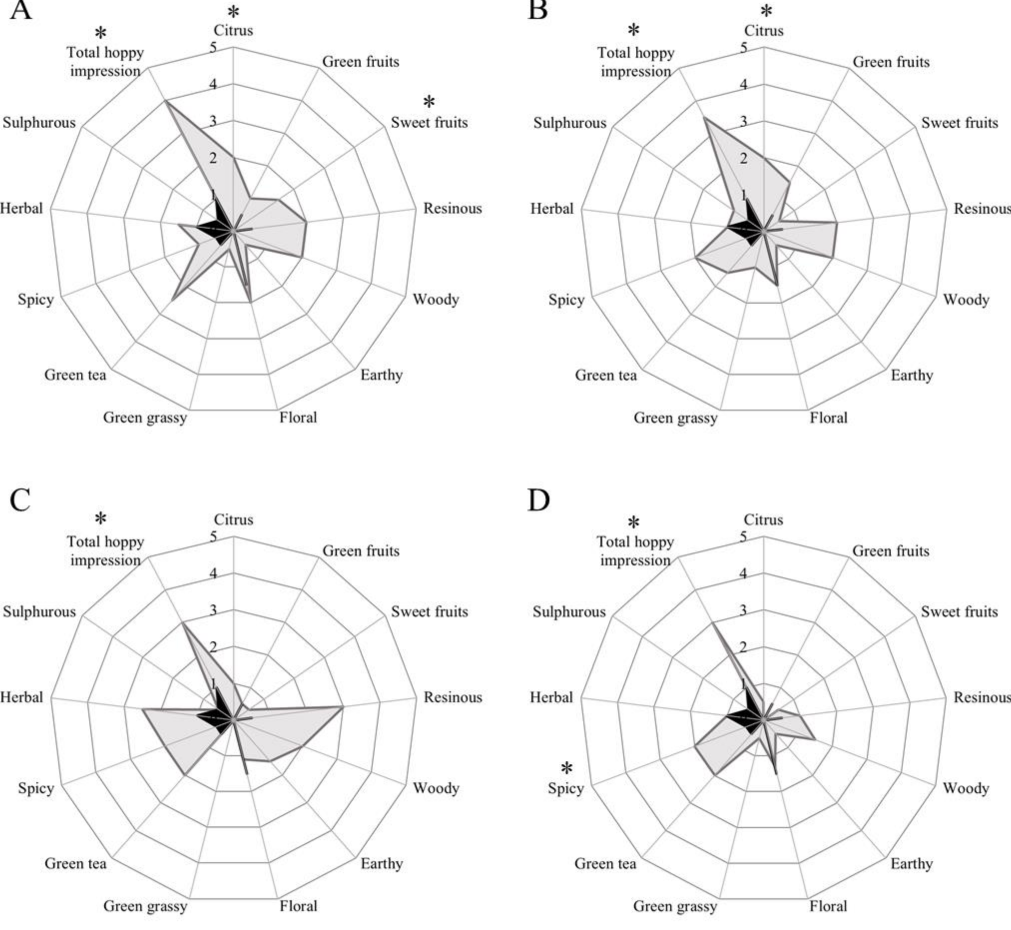


Figure 5.3. QDA sensory analysis of beers dry-hopped with Portuguese wild hops. Not dry-hopped beer scores are represented with dark grey area. **(A)** PTW2-beer (light grey area); **(B)** PTW7-beer; **(C)** PTW8-beer; **(D)** PTG22-beer. Data from quantitative descriptive analysis (QDA) performed by 10 trained panelists are presented as means (floral) or medians (remaining attributes). Values with the symbol * present significant statistic differences ($p < 0.05$) between descriptors mean in normal distribution and median in non-normal distribution. * *p* Values from Mann-Whitney analysis.

5.3.5. Quantification of compounds in beer

A high variety of volatile compounds can be found on hops and beers. Nevertheless, in commercial varieties, esters and terpenes have been noticed as the main classes of odour-active compounds of hops. Three esters, ethyl 2-methylpropanoate, ethyl 2-methylbutanoate, ethyl 4-methylpentanoate and three terpenes, myrcene, linalool and geraniol are among the most usually cited hop odour-active compounds (31, 60-62, 64, 68, 121, 122, 186, 187). Providing spicy, floral and citrus aroma, myrcene, linalool and geraniol have been proposed as markers for the intensity and the quality of hoppy aroma on beers (1, 33, 60, 61, 63, 211, 212). Furthermore, GC-O analysis also detected sulphur compounds as relevant of odour-active compounds in Portuguese hops. Three sesquiterpenes (β-caryophyllene, α-humulene and humulene oxide) that impart woody notes and are usually found in hops were also quantified in beers. Thus, selected key compounds quantified in beers, were ethyl 2-methylpropanoate, ethyl 2-methylbutanoate, ethyl 4-methylpentanoate, myrcene, linalool, geraniol, DMTS, S-methyl hexanthioate, β-caryophyllene, α-humulene, and humulene oxide. Results are presented in Figure 5.4. The minimal threshold levels found in literature for beer were also included in the graphics. Concerning beers dry-hopped with Portuguese hops, PTW2 and PTW7 beers increased ethyl-2-methylpropanoate above threshold levels, whereas ethyl-2-methylbutanoate was below threshold levels on all beers, and PTW2 was the only beer that reached the threshold levels of ethyl-4-methypentanoate and DMTS. Myrcene, linalool, and S-methyl hexanthioate were above threshold level in all dry-hopped beers, whereas geraniol was above threshold level only in PTW2 and PTG22 dry-hopped beers.

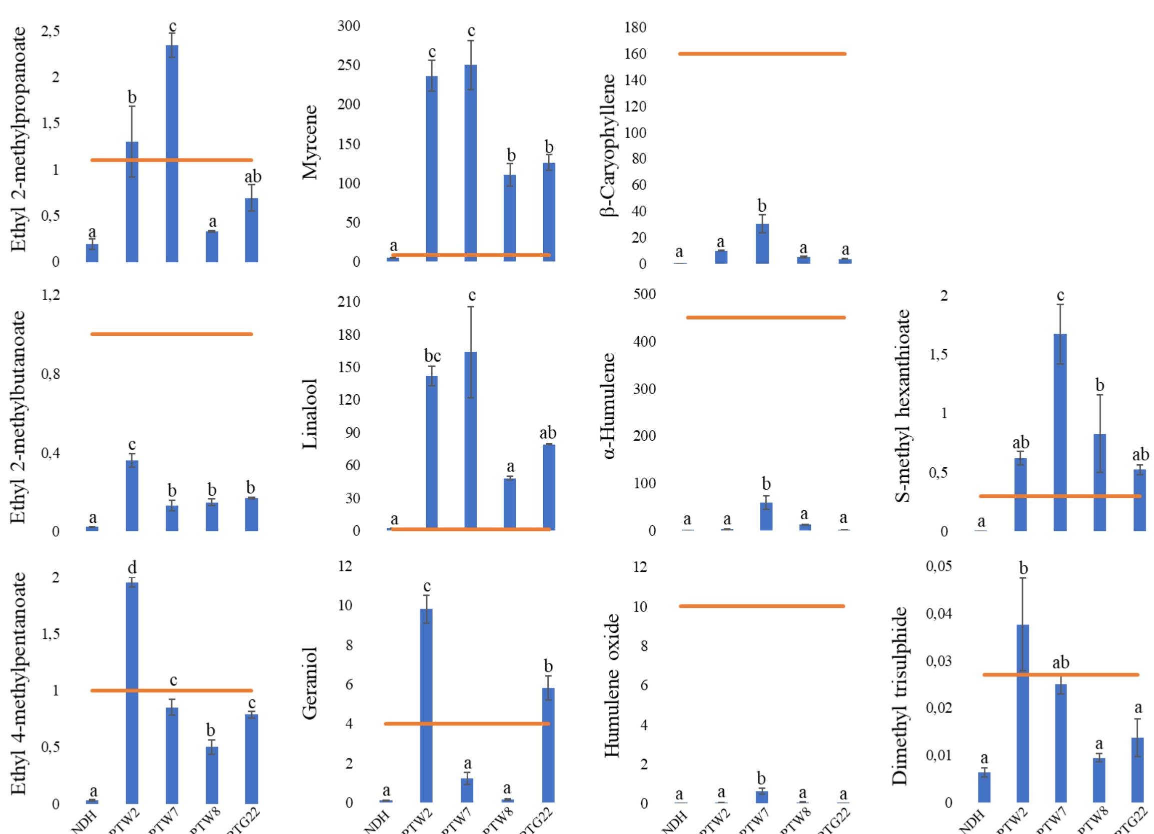


Figure 5.4. Quantification (µg/ L) of compounds in no dry-hopped (NDH) and beer dry-hopped with Portuguese wild hops. ANOVA test, using Tukey's post hoc test were performed to multiple pairwise comparison of samples. The orange line represents the lowest threshold determined in beer, from Hop Flavor Database of American Society of Brewing Chemists (213). Different letters for each compound show statistically significant differences ($p < 0.05$) between means.

In general, results are in agreement with the sensory analysis of beers, because PTW2 over PTW7 dry-hopped beers presented the most notorious fruity and hops impression. Esters from hops have been associated to fruity characteristic on beers (121, 214), whereas the content of monoterpenes has positive correlation with the sensory intensity of hoppy aroma (14). PTW2 hop enhanced the concentration of all tested esters and terpenes, while PTW7 promoted significant increase of myrcene, linalool, and two esters.

Concerning sulphurous compounds, PTW2 hop promoted a significant increase of dimethyl trisulphide, whereas in PTW7 and PTW8 dry-hopped beers, there was significant increase of S-methyl hexanthioate content. Nevertheless, no statistical differences were found in sulphurous attribute in QDA analysis of beers. The contents of β-caryophyllene, α-humulene and humulene oxide were below threshold levels in all dry-hopped beers, which justifies the low woody notes. Oxidation products of sesquiterpenes have been associated with spicy hop character in beer, however, the compounds so far identified have exhibited concentrations below their detection thresholds, and their aroma characteristics do not

correspond to the desired spicy hop aroma (61). No association was found between spicy notes of PTG22 dry-hopped beer and its volatile composition.

Taking in consideration threshold values, the quantification of key odour-active compounds in dry-hopped beers explains the high impact of PTW2 hop on dry-hopped beer sensory impression and the lower hoppy impression reported in PTW8 and PTG22 dry-hopped beers, which reflects the lower capacity of these hops to impart high concentration of key odour-active compounds to beers. However, the association between odour-active hop compounds and beer flavour results is not always clear because combinatory, competing, masking antagonistic and synergistic effects occur.

5.4. Conclusion

Four hops were selected due to their genetic diversity and different chemical composition. CATA analysis demonstrated that those four Portuguese hops differ in fruity (including citrus, green fruit, and sweet fruit), floral and woody impression. GC-O was a good approach to complement genetic, chemical (xanthohumol, alpha and beta acids) and sensorial characterization of wild hops. Throughout this methodology, 38 odour-active compounds, including the terpenes and esters, commonly related in commercial hops and a several sulphur compounds were identified. However, the evaluation of brewing characteristics of those hops cannot be predicted only by hops analyses, therefore sensory evaluation and quantification of key compounds on dry-hopped beers is also relevant information. Portuguese hops promoted fruity and spicy notes in dry-hopped beer and no unpleasant sulphurous sensation was observed. Concerning the Portuguese wild hop genotypes, PTW2 seems the most promising genotype.

CHAPTER 6

Prediction of fruity-citrus intensity of beers dry-hopped with Mandarina Bavaria

6.1. Introduction

Dry-hopping is a common practice of hops addition at the cold stage of brewing process (1). It is widely used in craft beers to impart intense hoppy flavour (13). A great number of volatile compounds are relevant for the hoppy flavour, including methyl butyl esters (fruity), ketones (fruity, citrus and floral), terpenes (herbal, woody, spicy and terpenic), methyl thioesters (sulphur and fruity), monoterpenoid alcohols (floral and citrus), monoterpenoid esters (fruity, greenery and floral), and cinnamate esters (fruity and balsamic) (1, 2, 12). Associations between hop esters and fruity characteristic of beers have been reported (121, 214), and correlation of monoterpenes with the sensory intensity of hoppy aroma are also described (14). However, empirical experience is the main factor to produce the dry-hopped beers, since there is scarce information about the factors that influence the reproducibility (and consistent product quality) of dry-hopping with flavour varieties.

Forster and Gahr (2013) published a study comparing the extraction of compounds in beers dry-hopped with 4 German “Special Flavour” hops (205). These are hop varieties with fruity, citrusy and floral characteristics that have been bred to satisfy the demand of craft brewers for strong differentiating hop-derived aroma and flavour notes to be used in dry-hopping beers. Mandarina Bavaria (MBA) variety is a “Special Flavor” variety released in 2013 derived from crosses of female of US Cascade variety and male of Huell wild hop-derived breeding line. It shows sensory similarities to the US cultivars CAS and CEN. MBA is described as fruity, with pronounced mandarin and citrus, combined with traditionally hoppy sensations. By dry-hopping, it potentially promotes hoppy basic notes with strong fruity-citrusy (215). In a dosage of 1.5 ml total oil/ hl of MBA, the average score of intensity of hop flavour reached 6.5, against 4.0 of a no dry-hopped control, in a scale from 1 (not or hardly noticeable) to 10 (most intensely) (205). Harvest dates also influence the analytical and sensory characteristics of top-fermented beer dry-hopped with 2.5 ml total oil/ hl of MBA, the intensity of the hop flavour (smell and taste) ranged from 2.5 and 3.5 (scale from 1-low to 5 high intensity) (216). However, more studies are needed concerning the transfer of volatile compounds during dry-hopping time and the changes that occur on sensory profile. The goal of the present work was to predict the impact of MBA on fruity-citrus intensity of dry-hopped beers through the composition of selected volatile compounds. Therefore, the evolution of 24 volatile compounds associated with hop flavour (216) (ethyl 2-methylpropanoate, ethyl 2-methylbutanoate, ethyl 3-methylbutanoate, ethyl 4-methylpentanoate, 3-methylbutyl 2-methylpropanoate; 2-methylbutyl 2-

methylpropanoate, myrcene, s-methyl 5-methylpentanthioate, s-methyl thiohexanoate, linalool, α-terpineol, citronellol, nerol, geraniol, methyl-Z-geranate, 2-undecanone, methyl-4Z-decenoate, methyl-E-geranate, ethyl dihydrocinnamate, geranyl acetate, β-caryophyllene, α-humulene, ethylcinnamate, humulene oxide) on two different base beers and the changes on sensory profile were followed during 15 days of dry-hopping, to understand the association between key hop volatiles and the sensory characteristics of beer, independently from beer initial characteristics.

6.2. Materials and methods

6.2.1. Chemical reagents

Ethyl 2-methylpropanoate (≥ 99%), ethyl 2-methylbutanoate (99%), ethyl 3-methylbutanoate (≥ 98), ethyl 2-methylpentanoate (internal standard, ≥ 99%), ethyl 4-methylpentanoate (≥ 97%), 3-methylbutyl 2-methylpropanoate (≥ 98%), 2-methylbutyl 2-methylpropanoate (≥ 97%), 2-undecanone (≥ 98%), myrcene (≥ 90%), β-caryophyllene (≥ 80%), α-humulene (≥ 96%), S-methyl 5-methylpentanthioate, S-methyl thiohexanoate, linalool (97%), α-terpineol (≥ 98,5%), citronellol (≥ 95%), nerol (≥ 97%), geraniol (≥ 99%), methyl-4Z-decenoate (≥ 95%), methyl-E-geranate, geranyl acetate (≥ 97%), ethyl dihydrocinnamate (≥ 98%), ethyl cinnamate (≥ 98%), humulene oxide (99%) were purchased from Sigma Aldrich (St. Louis, Mo., US).

6.2.2. Base beers

Two different commercial Munich-Style Helles, both from Freising (Germany), were the base beers for dry-hopping experiments. Beer A, a more hoppy beer, with the addition of HTR at the beginning of cooking, and HTR / SGD after the end of cooking, in the whirlpool stage. Beer B, single hopped with PER variety at beginning of cooking and 10 min before casting the wort.

6.2.3. Dry-hopping trials

The hops used in dry-hopping trials were pellets (Type 90) of the hop variety MBA (harvested in 2017, Hallertau, Germany). Hops composition was evaluated by Chair of Brewing and Beverage Technology of TUM School of Life Sciences Weihenstephan (Table 6.1).

Table 6.1. Hops parameters.

Parameter	Method	Value
α-acids	EBC 7.7	7.5 wt. %
β-acids	EBC 7.7	6.5 wt. %
Total polyphenols	EBC 7.14	150 mg/ L
Xanthohumol	EBC 7.15	0.7 wt. %
Total oil	EBC 7.10	1.2 mL/ 100 g

Dry-hopped beers were prepared in 10 L kegs (Cornelius Deutschland GmbH, Langenfeld, Germany). Hop bags containing 24 g of pellets of MBA were added to 8 L of beers (3 g/ L, corresponding a dosage of 3.6 ml total oil/ hl) and kept in agitation per 3, 6, 10 and 15 days at 4 ºC. All trials were done in triplicate. The quantity of hops and the procedure were determined considering the usual practices of breweries (36) and previous studies of extraction of volatile compounds by dry-hopping (19, 68).

6.2.4. Quantification of volatile compounds by headspace solid phase micro-extraction coupled with gas-chromatography mass-spectrometry (HS-SPME-GC-MS)

Vials of 20 ml with polypropylene caps (Butyl/ PTFE, Achroma, Mühlheim, Germany) and divinylbenzene/ carboxen/ polydimethylsiloxane (DVB/ CAR/ PDMS) SPME fibers 50/30 µm (Supelco/ Sigma Aldrich, Bellafonte, Penn., US) were used for headspace-solid phase micro-extraction (HS-SPME) of beer volatile compounds. Beer samples (5 g) were exposed to the fiber for 30 min at 40 °C. Chromatographic analyses were performed using a TRACE 1300 Ultra gas chromatography directly coupled with an ISQ QD single quadrupole mass spectrometer (ThermoScientific, Waltham, Mass., US).

Volatile compounds were desorbed in the injection port at 250 °C for 0.5 min in splitless mode. The GC was equipped with a Trace GOLD TG-5MS (ThermoScientific Waltham, Mass., US) 60 m × 0.25 mm × 0.25 mm capillary column. Oven temperature started at 60 °C, held for 4 min, increased at a rate of 5 °C/ min to 220 °C, held for 5 min, heated at a rate of 10 °C/ min to 250 °C, and held for 2 min. The transfer line was set to a temperature of 250 °C. The carrier gas helium BIP had a constant flow of 1.2 ml/ min. Measurements were done using the EI mode at an ionization energy of 70 eV. The mass spectrometer detected mass ranges between 35 and 350 u.

Calibration curves using internal standard (IS, ethyl 2-methylpentanoate 0.02 µg/ mL) were made on beer matrix (Munich-Style Helles). The identification of compounds was done with selection of the respective ions (showed in Tables 6.2 and 6.3) and comparing the retention indices and the mass spectra, with that of standards.

6.2.5. Sensory evaluation

Sensory evaluation was performed individually by a trained panel consisting of 25 participants (20–50 years of age) certified by the German agricultural society (Deutsche Landwirtschafts-Gesellschaft e.V.) for the analysis of beers. Tastings were carried out in standard cabins and fresh beer samples were blind-labelled with a three-digit code.

Beers were evaluated for total hoppy impression and fruity notes, including citrus, red berries, green fruits, and sweet fruits. Panellists had to analyse attributes by quantitative descriptive analysis (QDA) in scale of intensity from 0 to 5, where 0 = imperceptible, 1 = very weak, 2 = weak, 3 = middle, 4 = intensive, and 5 = very intensive.

6.2.6. Statistical analysis

All dependent variables, from volatile compounds quantification and QDA, were tested for distribution of the residuals with Shapiro–Wilk's test. When data presented normal distribution, t-Student' test was used to compare means of two samples, and one-way analysis of variance (ANOVA) was used for multiple comparison, with Tukey's or REGWQ test, since homogeneity of variances was, or was not, respectively confirmed by Levene's test.

Since normal distribution were not confirmed for QDA data, Maan-Whitney test was used for comparison of medians of two samples, and multiple pairwise comparisons by Kruskal-Wallis test, performed using Conover-Iman/ Two-tailed test.

Principle Component Analysis (PCA) was used to summarized data from volatile compounds quantification and to visualise the evolution of volatile compounds content during the 15 days of dry-hopping

Partial least squares (PLS) regression was used to study the relationships between beer sensory attributes (Y-matrix) and hops volatile profile (X-matrix) in terms of prediction of Y-variables from X-variables. Random validation was also applied to identify relevant X-variables.

All analysis was performed at 5% significance level using XLSTAT® for Windows version 2018.07 (Addinsoft, Paris, France).

6.3. Results and discussion

6.3.1. Evolution of volatile profile and sensory characteristics during dry-hopping

The two base beers presented significant differences in the concentration of 24 volatile compounds derived from hops, except the two thioesters quantified (Figure 6.1a), and distinct sensory profile for citrus, green fruits, and sweet fruits attributes (Figure 6.1b). Dry-hopping with MBA was performed on those two base beers.

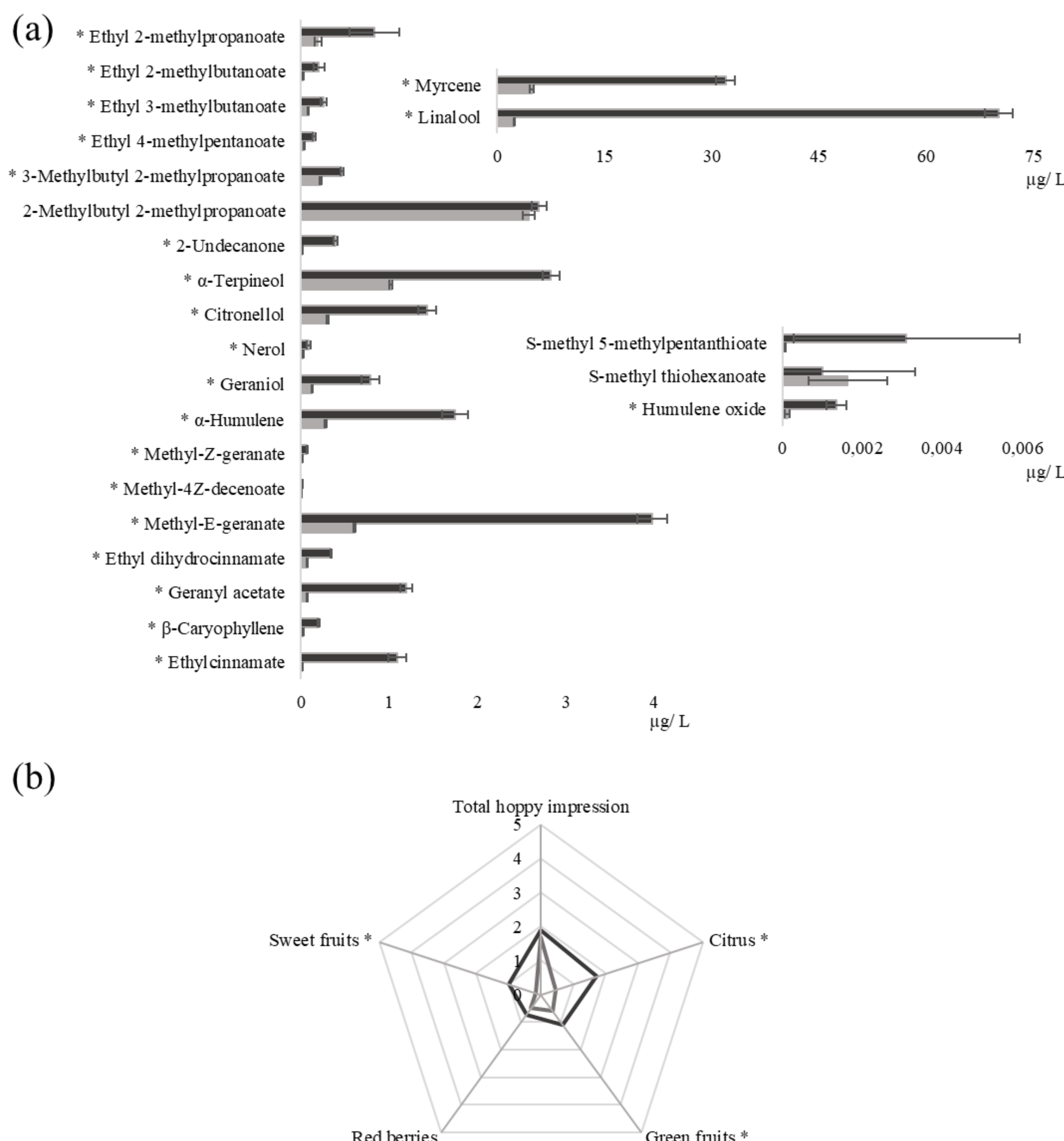


Figure 6.1. Summary of quantification of volatile compounds (a) and sensory evaluation (b) of two Munich-style Helles beers**.** Significant differences between beers was evaluated by t-student' test applied on data from quantification of volatile compounds (normal distribution), and Mann-Whitney test performed on sensory data (normal distribution was not found). * Symbol represent significant statistical differences ($p < 0.05$) between means or medians of beer A (dark colour) and beer B (light colour).

As expected, data from quantification of volatile compounds (Tables 6.2 and 6.3) pointed an increase of their content during dry-hopping. Myrcene, linalool, 2-methylbutyl 2-methylpropanoate (2MB2MP), α-humulene, and geraniol presented the highest concentration in all samples, which agrees with previous studies of dry-hopping with

different varieties (67). It should be highlighted that the initial beer composition influenced volatiles transfer, since in beer A, significant statistical differences occurred after days 3 and 6 (Table 6.2), whereas, in beer B, most compounds showed significant increase after the first day of dry-hopping (Table 6.3). The exception were methyl thioesters that presented a significant increase from the first day of dry-hopping on both beers.

Table 6.2. Concentrations and thresholds (Th) (µg/ L) of hops-derived volatile compounds in beer A before (day 0) and during 15 days of dry-hopping with 3 g/ L of Mandarina Bavaria hops.[1]

Beer A	ms/ RI	Day 0	Day 1	Day 3	Day 6	Day 10	Day 15	*p*-values	Th
Ethyl 2-methylpropanoate	116/ 753	0.8 (0.6 - 1.1) [d]	1.2 (1.1 - 1.8) [dc]	3.4 (2.3 - 5.8) [cb]	6.6 (5.4 - 10.6) [ba]	6.8 (6.7 - 13.1) [a]	12.5 - 15.4 [a]	0.012 [+]	1.1 - 5000
Ethyl 2-methylbutanoate	71/ 846	0.2 ± 0.1 [c]	0.5 ± 0.1 [cb]	0.8 ± 0.2 [cba]	1.7 ± 0.3 [a]	1.4 ± 0.4 [ba]	1.6 ± 0.9 [ba]	0.003 [#]	1.1
Ethyl 3-methylbutanoate	88/ 851	0.25 ± 0.03 [b]	0.37 ± 0.09 [b]	0.53 ± 0.09 [ba]	1.18 ± 0.21 [a]	0.84 ± 0.27 [ba]	1.14 ± 0.55 [a]	0.007 [#]	2 - 1300
Ethyl 4-methylpentanoate	88/ 964	0.15 (0.13 - 0.16) [c]	0.16 (0.13 - 0.21) [c]	0.23 (0.19 - 0.24) [cb]	0.37 - 0.37 [a]	0.31 (0.30 - 0.32) [ba]	0.26 - 0.36 [ba]	0.019 [+]	1 - 18
Myrcene	136/ 991	32 (31 - 33) [c]	62 (47 - 91) [cb]	124 (88 - 125) [ba]	76 (69 - 123) [cb]	147 (126 - 177) [a]	98 (77 - 112) [ba]	0.015 [+]	9 - 1000
3-Methylbutyl 2-methylpropanoate	71/ 1008	0.5 ± 0.01 [c]	4.8 ± 0.9 [cb]	7.3 ± 0.9 [ba]	11.6 ± 1.5 [a]	11.6 ± 1.3 [a]	9.8 ± 3.4 [a]	< 0.0001 [*]	> 30
2-Methylbutyl 2-methylpropanoate	71/ 1012	3 ± 0.1 [d]	30 ± 5 [c]	41 ± 3 [cb]	55 ± 6 [ba]	58 ± 6 [a]	48 ± 12 [ba]	< 0.0001 [*]	50 - 60
S-methyl 5-methylpentanthioate	131/ 1057	0.003 ± 0.003 [c]	0.10 ± 0.03 [ba]	0.15 ± 0.03 [ba]	0.10 ± 0.01 [cb]	0.19 ± 0.02 [a]	0.16 ± 0.07 [ba]	0.0003 [*]	15
S-methyl thiohexanoate	131/ 1093	0 (0 - 0.004) [c]	0.11 (0.06 - 0.14) [ba]	0.13 (0.11 - 0.23) [a]	0.058 (0.056 - 0.065) [cb]	0.10 (0.10 - 0.12) [ba]	0.07 (0.03 - 0.09) [cba]	0.020 [+]	0.3 - 0.1
Linalool	80/ 1099	70 ± 2 [d]	84 ± 3 [dc]	90 ± 4 [cb]	115 ± 5 [a]	95 ± 4 [cb]	105 ± 12 [ba]	< 0.0001 [*]	1 - 100
α-Terpineol	121/ 1199	2.8 ± 0.1 [d]	3.6 ± 0.3 [dc]	3.9 ± 0.02 [cb]	6.1 ± 0.6 [a]	4.3 ± 0.5 [cb]	4.9 ± 0.4 [b]	< 0.0001 [*]	330 - 2000
Citronellol	69/ 1229	1.4 ± 0.1 [b]	1.9 ± 0.2 [ba]	1.6 ± 0.1 [ba]	2.2 ± 0.3 [a]	2.0 ± 0.5 [ba]	2.1 ± 0.2 [ba]	0.021 [#]	9 - 40
Nerol	69/ 1231	0.09 (0.06 - 0.10) [c]	0.29 (0.22 - 0.34) [cb]	0.26 (0.29 - 0.37) [cb]	0.58 (0.45 - 0.59) [a]	0.40 (0.30 - 0.46) [ba]	0.38 (0.30 - 0.55) [ba]	0.019 [+]	80 - 500
Geraniol	69/ 1257	0.8 ± 0.1 [c]	8.6 ± 2.1 [cb]	10.7 ± 1.6 [cb]	25.6 ± 6.6 [a]	18.8 ± 4.6 [ba]	19.4 ± 10.6 [ba]	0.001 [#]	4 - 500
Methyl-Z-geranate	114/ 1283	0.06 ± 0.002 [d]	0.09 ± 0.01 [dc]	0.10 ± 0.01 [dcb]	0.17 ± 0.04 [ba]	0.17 ± 0.02 [a]	0.15 ± 0.04 [cba]	0.0003 [*]	NIF
2-Undecanone	170/ 1300	0.4 ± 0.02 [d]	2.7 ± 1.1 [dc]	4.3 ± 0.8 [cb]	6.3 ± 1.7 [ba]	8.5 ± 0.8 [a]	6.1 ± 1.7 [ba]	< 0.0001 [*]	400
Methyl-4Z-decenoate	152/ 1310	0.003 (0.003 - 0.003) [c]	8.25 - 8.31 [cb]	13.9 - 15.8 [ba]	11.5 - 15.7 [ba]	20.0 - 21.1 [a]	11.1 - 15.0 [cba]	0.053 [+]	200 - 300
Methyl-E-geranate	114/ 1325	4.0 ± 0.2 [d]	6.8 ± 1.3 [dc]	8.2 ± 0.5 [cb]	10.8 ± 2.1 [ba]	12.2 ± 1.3 [a]	10.1 ± 1.9 [cba]	0.0001 [*]	NIF
Ethyl dihydrocinnamate	178/ 1357	0.34 (0.33 - 0.34)	0.40 (0.36 - 0.42)	0.33 (0.32 - 0.40)	0.43 (0.27 - 0.45)	0.40 (0.22 - 0.40)	0.36 (0.27 - 0.40)	0.637 [+]	NIF
Geranyl acetate	93/ 1385	1.2 ± 0.1 [d]	1.4 ± 0.1 [dc]	1.6 ± 0.1 [dcb]	2.0 ± 0.3 [cba]	2.3 ± 0.3 [ba]	2.4 ± 0.4 [a]	0.0002 [*]	NIF
β-Caryophyllene	133/ 1442	0.2 ± 0.01 [c]	2.8 ± 0.9 [cb]	7.0 ± 0.6 [b]	8.0 ± 4.30 [b]	14.9 ± 2.6 [a]	7.6 ± 3.8 [b]	0.0002 [#]	160 - 450
α-Humulene	80/ 1476	2 ± 0.1 [c]	12 ± 2 [cb]	24 ± 2 [b]	27 ± 9 [b]	43 ± 6 [a]	25 ± 9 [b]	< 0.0001 [#]	50 - 630
Ethylcinnamate	176/ 1482	1.1 ± 0.1 [c]	3.8 ± 0.7 [cb]	7.1 ± 0.4 [b]	7.9 ± 2.6 [b]	12.5 ± 1.7 [a]	7.1 ± 2.1 [b]	< 0.0001 [*]	NIF
Humulene oxide	138/ 1633	0.001 (0.001 - 0.002) [d]	0.04 - 0.10 [dc]	0.19 (0.13 - 0.23) [cba]	0.30 - 0.34 [a]	0.21 (0.15 - 0.28) [ba]	0.12 - 0.13 [dcb]	0.026 [+]	10 - 450

Table 6.3. Concentrations and thresholds (Th) (µg/ L) of hops-derived volatile compounds in beer B before (day 0) and during 15 days of dry-hopping with 3 g/ L of Mandarina Bavaria hops. [1]

Beer B	ms/ RI	Day 0	Day 1	Day 3	Day 6	Day 10	Day 15	*p*-values	Th
Ethyl 2-methylpropanoate	116/ 753	0.19 (0.15 - 0.23) ^{e}	0.6 (0.4 - 0.6) ^{d}	1.1 (0.9 - 1.1) ^{c}	4.0 - 5.1 ^{cb}	6.2 - 7.0 ^{ba}	10.2 - 10.3 ^{a}	0.018 $^{+}$	1.1 - 5000
Ethyl 2-methylbutanoate	71/ 846	0.02 ± 0.002 ^{b}	0.1 ± 0.02 ^{b}	0.3 ± 0.1 ^{b}	0.7 ± 0.2 ^{a}	1.0 ± 0.4 ^{a}	1.1 ± 0.1 ^{a}	0.0004 $^{\#}$	1.1
Ethyl 3-methylbutanoate	88/ 851	0.08 (0.07 - 0.09) ^{b}	0.07 (0.06 - 0.11) ^{b}	0.09 (0.07 - 0.14) ^{b}	0.15 (0.13 - 0.23) ^{ba}	0.16 - 0.21 ^{ba}	0.25 (0.23 - 0.28) ^{a}	0.024 $^{+}$	2 - 1300
Ethyl 4-methylpentanoate	88/ 964	0.03 ± 0.004 ^{c}	0.15 ± 0.03 ^{b}	0.15 ± 0.01 ^{b}	0.26 ± 0.04 ^{a}	0.30 ± 0.02 ^{a}	0.15 ± 0.02 ^{b}	< 0.0001 *	1 - 18
Myrcene	136/ 991	4.8 ± 0.3 ^{b}	88 ± 19 ^{a}	63 ± 13 ^{a}	85 ± 20 ^{a}	91 ± 25 ^{a}	88 ± 16 ^{a}	0.0004 *	9 - 1000
3-Methylbutyl 2-methylpropanoate	71/ 1008	0.2 ± 0.01 ^{c}	3.6 ± 0.4 ^{b}	3.5 ± 0.6 ^{b}	5.5 ± 0.8 ^{a}	6.5 ± 1.0 ^{a}	6.8 ± 0.3 ^{a}	< 0.0001 *	> 30
2-Methylbutyl 2-methylpropanoate	71/ 1012	2.6 ± 0.1 ^{c}	22 ± 2 ^{b}	21 ± 3 ^{b}	28 ± 2 ^{a}	30 ± 3 ^{a}	30 ± 1 ^{a}	< 0.0001 $^{\#}$	50 - 60
S-methyl 5-methylpentanthioate	131/ 1057	0.0001 ± 2E-06. ^{b}	0.05 ± 0.01 ^{a}	0.03 ± 0.01 ^{ba}	0.07 ± 0.02 ^{a}	0.06 ± 0.02 ^{a}	0.04 ± 0.01 ^{a}	0.001 *	15
S-methyl thiohexanoate	131/ 1093	0.002 (0.001 - 0.003) ^{b}	0.03 (0.03 - 0.04) ^{a}	0.02 (0.01 - 0.02) ^{ba}	0.04 (0.02 - 0.05) ^{a}	0.03 (0.02 - 0.03) ^{ba}	0.02 (0.01 - 0.02) ^{ba}	0.030 $^{+}$	0.3 - 0.1
Linalool	80/ 1099	2 ± 0.01 ^{c}	22 ± 2 ^{b}	24 ± 3 ^{b}	32 ± 3 ^{a}	36 ± 3 ^{a}	36 ± 5 ^{a}	< 0.0001 *	1 - 100
α-Terpineol	121/ 1199	1.0 (1.0 - 1.0) ^{a}	3.0 (2.6 - 3.1) ^{bc}	2.4 (2.2 - 2.5) ^{ab}	3.2 (2.7 - 3.8) ^{bcd}	3.6 (3.4 - 4.3) ^{cd}	4.7 (3.3 - 4.7) ^{d}	0.011 $^{+}$	330 - 2000
Citronellol	69/ 1229	0.3 ± 0.01 ^{b}	0.6 ± 0.1 ^{a}	0.6 ± 0.02 ^{a}	0.6 ± 0.1 ^{a}	0.7 ± 0.1 ^{a}	0.7 ± 0.2 ^{a}	0.0002 $^{\#}$	9 - 40
Nerol	69/ 1231	0.01 ± 0.001 ^{c}	0.2 ± 0.1 ^{cb}	0.2 ± 0.1 ^{cb}	0.4 ± 0.1 ^{ba}	0.5 ± 0.1 ^{a}	0.7 ± 0.2 ^{a}	0.003 $^{\#}$	80 - 500
Geraniol	69/ 1257	0.1 (0.1 - 0.1) ^{c}	3.1 (2.7 - 3.5) ^{cb}	3.2 (2.3 - 3.4) ^{cb}	6.6 (4.6 - 9.4) ^{ba}	8.0 (7.0 - 13.1) ^{a}	17.4 (10.7 - 17.4) ^{c}	0.008 $^{+}$	4 - 500
Methyl-Z-geranate	114/ 1283	0.01 ± 0.0003 ^{d}	0.07 ± 0.01 ^{cb}	0.05 ± 0.01 ^{c}	0.08 ± 0.02 ^{cba}	0.10 ± 0.02 ^{ba}	0.12 ± 0.01 ^{a}	< 0.0001 *	NIF
2-Undecanone	170/ 1300	0.01 ± 0.001 ^{d}	2.2 ± 0.8 ^{cb}	1.1 ± 0.4 ^{dc}	2.1 ± 0.8 ^{cb}	2.8 ± 0.8 ^{ba}	4.0 ± 0.2 ^{a}	< 0.0001 *	400
Methyl-4Z-decenoate	152/ 1310	0.0002 ± 0.0001 ^{b}	10.1 ± 3.1 ^{a}	5.3 ± 1.6 ^{a}	8.4 ± 2.0 ^{a}	9.3 ± 1.6 ^{a}	9.8 ± 0.6 ^{a}	< 0.0001 $^{\#}$	200 - 300
Methyl-E-geranate	114/ 1325	0.6 ± 0.01 ^{d}	3.9 ± 0.6 ^{cb}	2.9 ± 0.5 ^{c}	4.2 ± 0.9 ^{cb}	4.9 ± 0.9 ^{ba}	5.8 ± 0.1 ^{a}	< 0.0001 *	NIF
Ethyl dihydrocinnamate	178/ 1357	0.1 (0.1 - 0.1)	0.3 (0.2 - 0.3)	0.2 (0.2 - 0.3)	0.3 (0.2 - 0.4)	0.3 (0.2 - 0.3)	0.3 (0.2 - 0.3)	0.078 $^{+}$	NIF
Geranyl acetate	93/ 1385	0.1 ± 0.001 ^{d}	0.4 ± 0.01 ^{c}	0.4 ± 0.01 ^{c}	1.0 ± 0.2 ^{a}	0.6 ± 0.1 ^{b}	0.9 ± 0.04 ^{a}	< 0.0001 $^{\#}$	NIF
β-Caryophyllene	133/ 1442	0.02 (0.02 - 0.02) ^{b}	6.4 (3.5 - 6.4) ^{a}	3.0 (1.6 - 3.5) ^{ba}	4.2 (2.9 - 5.0) ^{ba}	5.1 (3.8 - 5.9) ^{a}	5.9 (5.1 - 6.8) ^{a}	0.027 $^{+}$	160 - 450
α-Humulene	80/ 1476	0.3 (0.3 - 0.3) ^{b}	19 (12 - 19) ^{a}	11 (6 - 12) ^{ba}	13 (10 - 15) ^{ba}	15 (12 - 17) ^{a}	16 (15 - 18) ^{a}	0.030 $^{+}$	50 - 630
Ethylcinnamate	176/ 1482	0.01 ± 0.01 ^{c}	4.3 ± 1.2 ^{ba}	2.4 ± 0.8 ^{b}	3.3 ± 0.7 ^{ba}	4.1 ± 0.8 ^{ba}	4.6 ± 0.6 ^{a}	< 0.0001 *	NIF
Humulene oxide	138/ 1633	0.0001 ± 0.00002 ^{c}	0.04 ± 0.02 ^{ba}	0.01 ± 0.004 ^{cb}	0.03 ± 0.02 ^{cba}	0.02 ± 0.01 ^{cb}	0.06 ± 0.02 ^{a}	0.013 *	10 - 450

[1]In Tables 6.2 and 6.3 data modeled by normal distribution (expressed as mean ± standard deviation; n = 3.) were evaluated by: * one-way ANOVA Tukey's test or # one-way ANOVA REGWQ test. + Comparison K samples with Conover-Iman test, were performed for data without normal distribution, expressed as median (minimum - maximum). Different letters in a row show statistically significant differences, at *p*-values, between means and medians. ms/ RI = fragment mass and retention index. Thresholds in beer retrieved from Hop Flavor Database (http://methods.asbcnet.org/hop_Flavors_Database.aspx). NIF = not in file.

PCA was applied on data from quantification of volatile compounds in beer samples during dry-hopping (from day 1 to day 15) to understand the relationship between the concentration of each compound and the time of dry-hopping (Figure 6.2). It is possible to observe that, in beer A, highest volatile concentration occurred at days 6 and 10, whereas, in beer B, it was latter at days 10 and 15. In general, methyl and methyl butyl esters, together with monoterpenoid alcohols were the compounds that presented maximum concentration up to day 6 in beer A and day 10 in beer B.

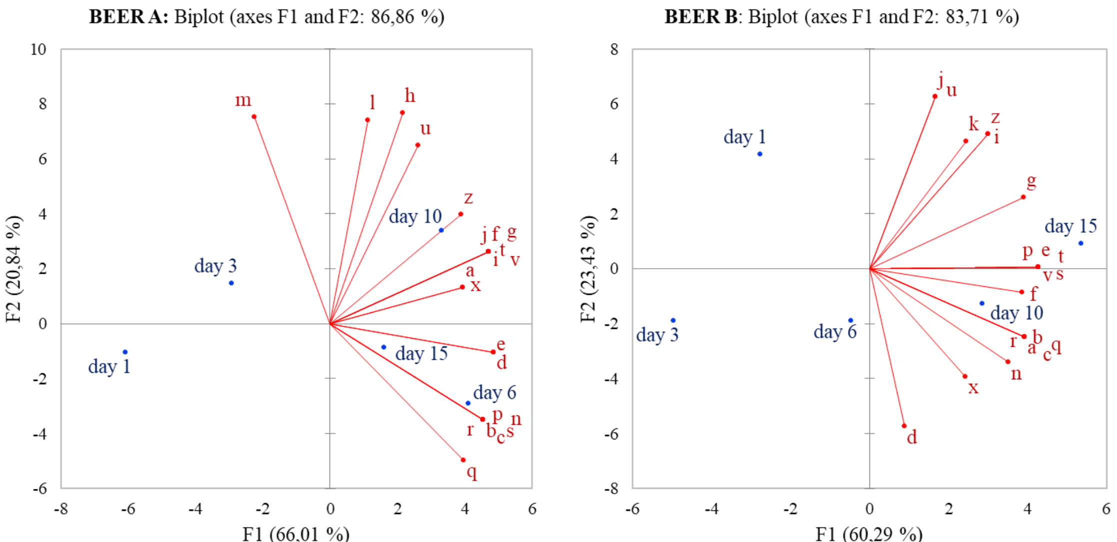


Figure 6.2. Principal Component Analysis (PCA) of the relation of days of dry-hopping (blue) and the extraction of compounds (red) for beer A and B. Compounds are represented by letters: (a) ethyl 2-methylpropanoate, (b) ethyl 2-methylbutanoate, (c) ethyl 3-methylbutanoate, (d) ethyl 4-methylpentanoate, (e) 3-methylbutyl 2-methylpropanoate, (f) 2-methylbutyl 2-methylpropanoate, (g) 2-undecanone, (h) myrcene, (i) β-caryophyllene, (j) α-humulene, (k) humulene oxide, (l) S-methyl 5-methylpentanthioate, (m) S-methyl thiohexanoate, (n) linalool, (p) α-terpineol, (q) citronellol, (r) nerol, (s) geraniol, (t) methyl-Z-geranate, (u) methyl-4Z-decenoate (v) methyl-E-geranate, (x) geranyl acetate, (z) ethylcinnamate.

Concerning the evolution of sensory profile during dry-hopping, the scores given by panellists increased in most descriptors, namely, total hoppy aroma intensity, citrus, green fruits, and sweet fruits but not of red berries (Figure 6.3). In beer A, statistical differences were observed from day 1, for total hoppy and sweet fruit, and day 3, for citrus and green fruits. In beer B, statistical significant differences were noticed from the day 3 for all descriptors.

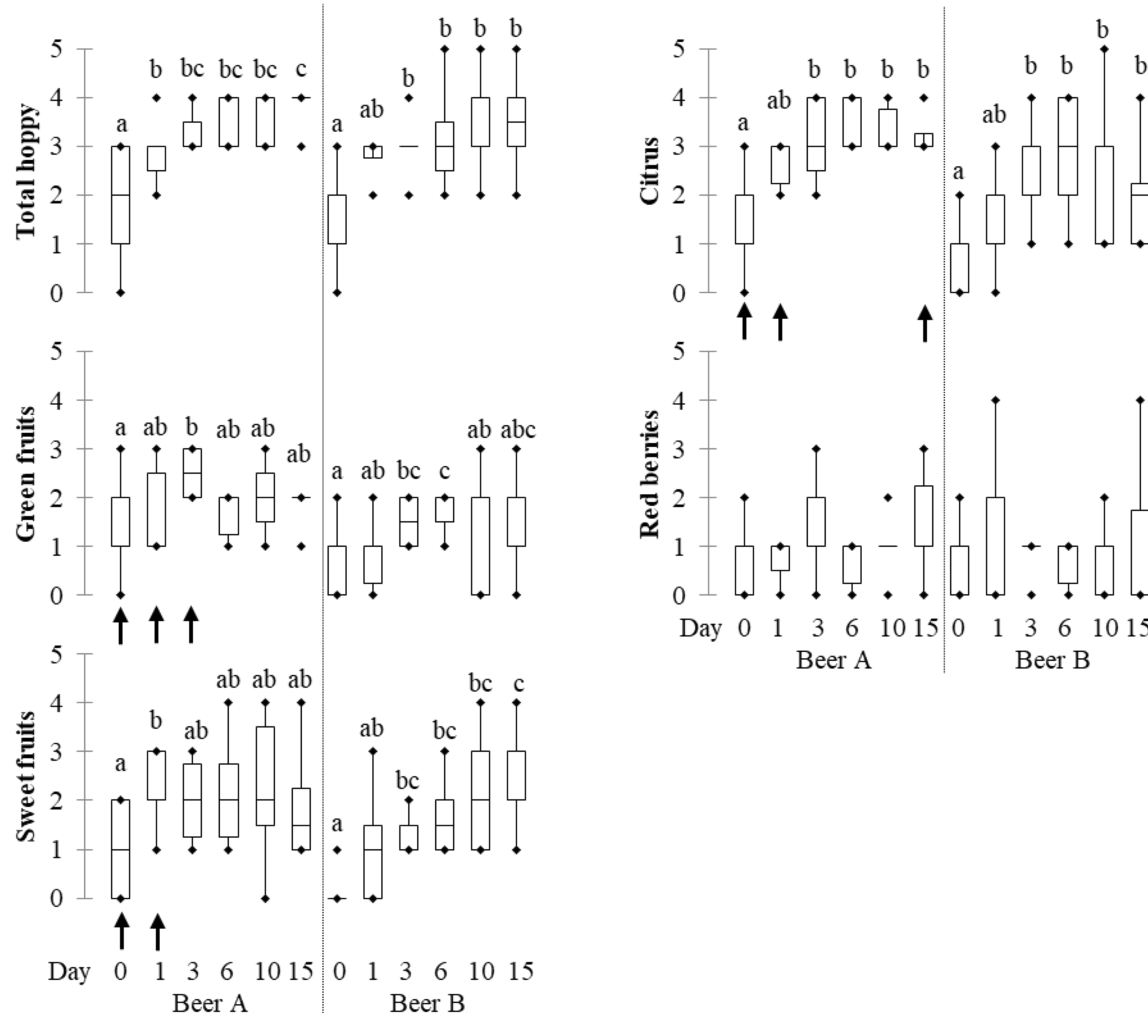


Figure 6.3. Sensory evaluation of two different base beers before and after 1, 3 6, 10, and 15 days of dry-hopping with Mandarina Bavaria hops (3 g/ L). Sensory analysis was performed by 25 trained panellists by quantitative descriptive analysis (QDA). Normality test was applied. Data are presented by boxplots including medians, quartiles, minimum and maximum values, since samples were not modelled by normal distribution. Multiple pairwise comparisons by Kruskal-Wallis test, performed using Conover-Iman/ Two-tailed test were performed to compare data from the same beer with different time of dry-hopping. Different letters represent significant differences for each attribute (respectively, in beer A and beer B, p-values < 0.0001 and 0.001 for citrus; 0.002 and 0.034 for green fruits; 0.167 and 0.837 for red berries; 0.006 and < 0.0001 for sweet fruits; and < 0.0001 and 0.001 for total hoppy impression). Mann-Whitney were performed to compare data from beers A and B, at the same time of dry hopping (arrows represent significant differences).

The comparison between both beers (A and B) at day 1 pointed significant differences for citrus, sweet fruits, and green fruits descriptors, whereas, at day 3, beers significant differences were observed only for green fruits descriptor. From day 6 to 10, no significant statistical differences were found, although, after 15 days of dry-hopping, beer A presented higher intensity of citrus descriptor.

The relationship between hop volatile compounds and sensory perception of hops characteristics in dry-hopped beers has been addressed taking in consideration thresholds and odour description of the hops-derived compounds (1, 67, 205) and concerning the hop MBA, scarce studies were found. Nevertheless, when Forster and Gahr (2013) compared the extraction of volatile compounds in beers dry-hopped with 4 German special flavour varieties, including MBA, they observed a significant increase in concentrations of linalool, geraniol, myrcene, and typical hop esters, namely, 3-methylbutyl 2-methylpropanoate and 2MB2MP, reaching thresholds values (205). Additionally, authors suggested that the analysis of other compounds that were not evaluated, like nerol, citronellol, and methyl geranate would be interesting. More recently, (216) described that beers dry-hopped with MBA presented ethyl 2-methylpropanoate (sweet, fruity), ethyl 2-methylbutanoate (fruity), ethyl 4-methylpentanoate (ester-like, fruity, sour), linalool (fruity, flowery) and geraniol (flowery, orange peel) as hop-derived aroma-active compounds. Odour description was evaluated by olfactometry. Surprisingly, in this study, authors did not identify myrcene. All these volatile compounds were quantified in this study. However, only myrcene, linalool, 2MB2MP, geraniol, ethyl 2-methylpropanoate and ethyl 2-methylbutanoate, reached threshold values (Table 6.2 and 6.3).

Beside volatiles thresholds and odour description, chemometric techniques were shown as relevant tools to study the relationship between volatile profile and sensory perception (68). These authors evaluated beers dry-hopped with COM variety and present a heatmap with the correlation between tasting results and the quantification of volatile compounds. The highest sensation of hoppy attributes (including fruity and citrus) was correlated with the highest content of nine compounds originated from hops. However not all of those compounds were present in concentrations higher than their threshold (68). A model that predicts the sensory characteristics of dry-hopped beers taking in consideration the volatile compounds content is still lacking.

6.3.2. Prediction of sensory characteristics of dry-hopped beers by quantification of select volatiles

Partial Least Squares regressions (PLS-R) was done to explore the relationship between sensory descriptors and the quantified 24 volatile compounds, regardless of the beer sample. For this regression, only sensory descriptors and volatile compounds with statistically significant differences were considered. Regression models were considered successful when cumulative variation of X and Y explained in terms of sum of squares (R^2X and R^2Y) were equal or superior to 0.70, and presenting good ability to predict new samples

when cumulative predictive variation from internal cross-validation (Q^2) was equal or superior 0.50 (201).

Successful models (cumulative values Q^2 = 0.68, R^2X = 0.75 and R^2Y = 0.71) were obtained for all sensory descriptors. Values of variable importance in projection (VIP) were calculated and compounds with VIP ≥ 0.80 were selected to further explore sensory characteristics prediction. Therefore, myrcene (VIP = 3.18), linalool (VIP = 2.80), 2MB2MP (VIP = 1.67), and α-humulene (VIP = 0.96) (Figure 6.4) could be considered to have moderate to high influence on the relationship with attributes perception (193, 194).

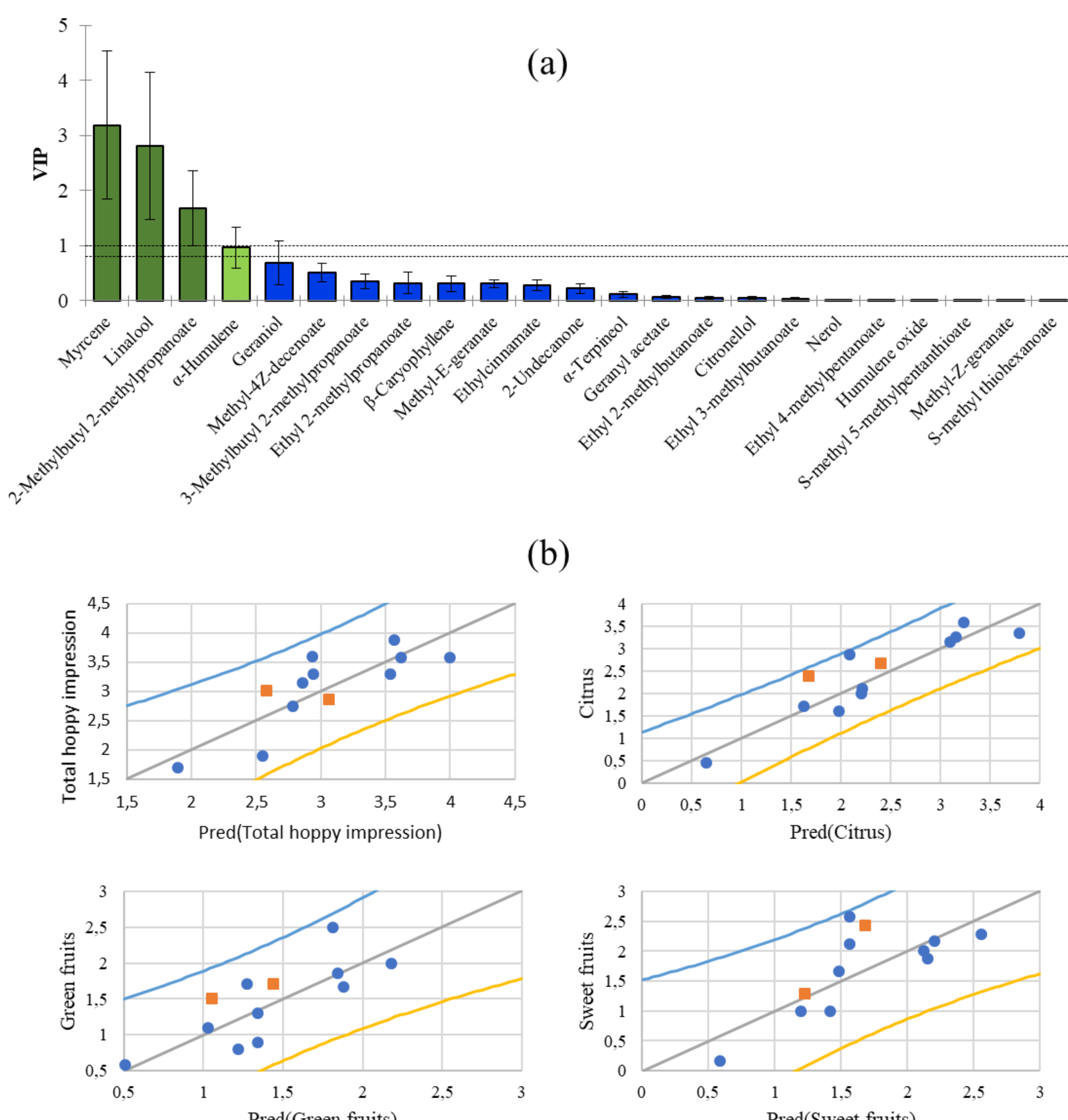


Figure 6.4. Prediction of sensory characteristics of dry-hopped beers by quantification of volatiles compounds. (a) Values of variable importance for the projection (VIP). Results were obtained by PLS-R with 95% confidence interval. Highly influential latent variables (dark green) have VIP > 1; moderately influential latent variables (light green) have 0.8 < VIP < 1; and latent variables with low influence have VIP < 0.8 (blue). (b) Predicted versus active standardized scores for the different sensory attributes. Active scores (●) used for prediction, and residuals scores (■) corresponding to the observations of the validation of the model.

Prediction of sensory characteristics (in a scale from 0 total 5) of total hoppy (Q^2 = 0.654), citrus (Q^2 = 0.745), greens fruits (Q^2 = 0.598), and sweet fruit (Q^2 = 0.626) can be estimated using equations resulting from PLS-R carried out with myrcene, linalool, 2MB2MP, and α-humulene (Table 6.4). For example, if beer composition is: myrcene = 100 µg/ L; 2MB2MP = 50 µg/ L; linalool = 80 µg/ L, and α-Humulene = 30 µg/ L the predicted score for Total

hoppy is 3.4 (Total hoppy = 1.8 + (100 × 7.5 × 10^{-3}) + (50 × 4.2 × 10^{-3}) + (80 × 7.2 × 10^{-3}) + (30 × 2.3 × 10^{-3})).

Table 6.4. Equations to predict attributes sensation using quantification of compounds with highly influential latent variables (VIP > 1) from the model.

Attribute sensation		Compound quantification (µg/ L)				
		Myrcene	**2MB2MP**	**Linalool**	**α-Humulene**	
Total hoppy	= 1.8	+ 7.5 × 10^{-3}	+ 4.2 × 10^{-3}	+ 7.2 × 10^{-3}	+ 2.3 × 10^{-3}	Q^2 0.654 R^2Y 0.728 SD 0.392 RSME 0.351
Citrus	= 0.7	+ 10.7 × 10^{-3}	+ 6.0 × 10^{-3}	+ 10.2 × 10^{-3}	+ 3.3 × 10^{-3}	Q^2 0.745 R^2Y 0.816 SD 0.435 RSME 0.389
Green fruits	= 0.6	+ 4.9 × 10^{-3}	+ 2.7 × 10^{-3}	+ 4.6 × 10^{-3}	+ 1.5 × 10^{-3}	Q^2 0.598 R^2Y 0.627 SD 0.321 RSME 0.287
Sweet fruits	= 0.4	+ 7.5 × 10^{-3}	+ 4.2 × 10^{-3}	+ 7.1 × 10^{-3}	+ 2.3 × 10^{-3}	Q^2 0.626 R^2Y 0.698 SD 0.422 RSME 0.377

Results obtained by Partial Least Squares regressions (PLS-R) between volatile composition (X-variables) and sensory attributes (Y-variables). Q^2, cumulative predictive variation from internal cross-validation; R^2, cumulative variation of Y explained in terms of sum of squares; SD, Standard deviation; RMSE, Root mean square error.

This is the first study presenting a model that predicts beer sensory characteristics taking in consideration volatile compounds content. Some compounds important to the model occurred in concentrations higher than threshold, such as myrcene, linalool, and 2MB2MP. However, α-humulene, demonstrated to be an important compound to predict beer sensory characteristics, even though did not reached threshold values (Table 6.2 and 6.3). In the other side, geraniol and the esters ethyl 2-methylpropanoate and ethyl 2-methylbutanoate presented concentrations higher than threshold (but were not important to the model). Nevertheless, the observed correlations do not necessarily imply causality. For that reason, correlation models obtained should be interpreted as showing associations rather than direct cause and effect relationship.

6.4. Conclusion

Two beers, with different volatile and sensory profiles were dry-hopped during 15 days with the variety MBA. The initial composition of the beer influenced the extraction of volatile compounds during dry-hopping. The beers with higher content of volatile compounds required more time to present significant statistical differences in volatiles increase. However, the highest volatile concentration occurred earlier in this beer. Concerning sensory profile, there was an increase of total hoppy, citrus, green fruits, and sweet fruits intensities in both beers. Nevertheless, although beers presented statistical differences in sensory profile at the beginning, these differences were fainter after 3 days of dry-hopping, up to 10 days, whereas at day 15, significant difference in citrus descriptor was noted again.

Successful models were obtained to predict total hoppy, citrus, greens fruits, and sweet fruit characteristics by equation regressions considering only the content of four selected volatile compounds, myrcene, 2-methylbutyl 2-methylpropanoate, linalool, and α-humulene.

It is important to observe that this study was done with one specific hop variety, MBA, used as a model of fruity-citrus varieties. The use of other varieties, particularly the ones with very different aroma profiles, will induce different sensorial perception, which may result in different model equations, due to the complexity of aroma interactions.

PART IV

Impact of hops in beer bioactivity

CHAPTER 7

Maximized extraction of α-acids and xanthohumol in dry-hopped beers

7.1. Introduction

Xanthohumol (XN) and α-acids received increased attention from researchers during the last years, due to their impact in beer sensory characteristics and their recognized bioactive activities, namely, antioxidant, antimicrobial and sedative proprieties. Therefore, XN and α-acids have been linked with beneficial health effects and regulation of some diseases (21, 22, 51).

XN have demonstrated positive effects against obesity and diabetes by regulation of glucose and cholesterol metabolism (217). XN and α-acids have also presented bioactivity as suppressors of osteoporosis with positive effects on balance between bone formation and bone resorption (218).

The dry-hopping technique is becoming more and more popular in commercial breweries, not only because it imparts a special hop aroma and bitterness to beer, but also because the increases amount of XN and α-acids content in the finished beer (219) influencing beer bioactivity. Although many different dry-hopping techniques exist, their poor reproducibility is the main disadvantage (220). Thus, tools to increase the productivity of the dry-hop usage are still required. Optimized dry hopping techniques and some processes for the XN enrichment of beers have been proposed (221) with the aim of preserving the beneficial phytochemicals present in hop. However, the hop content in bioactive compounds even from the same variety and growing region varies significantly, which requires studies concerning hop compounds extraction, in order to have an effective control over the process, as well as, to predict hops utilization and increase beer bioactivity.

The extraction of hop compounds to beer is known to be determined by several factors, namely the hop variety, quantity and form, extraction time and temperature, hop dispersion methods (static/dynamic), beer and yeast type (222). However, little is known about how to optimize and predict the extraction of these compounds. In this context, the aim of this study was to optimize the extraction time and hop quantity to reach the highest yield of extraction of XN and α-acids in beers by the dry-hopping technique. For that purpose, three of the most used worldwide hop varieties Chinook (CHI), East Kent Goldings (EKG) and Tettnanger (TET), as pellets type 90 representing different origins and α-acids contents were assayed at different dry-hopping concentration (during maturation) and time of extraction, and robust statistical approaches were used for data interpretation.

7.2. Materials and methods

7.2.1. Chemicals and materials

Beer ingredients as malts Best Pale Ale, 5 – 7 EBC (Bestmalz, Heidelberg, Germany) and Caramünch® III, 140 – 160 EBC, (Weyermann®, Bamberg, Germany), hops pellets type 90 from the varieties Summit (SUM) (Yakima Valley Hops, Yakima, US), CHI (Yakima Valley Hops, Yakima, US), EKG (Charles Faram, Malvern, United Kingdom) and TET (Charles Faram, Malvern, United Kingdom), and yeast M36 Liberty Bell Ale (Mangrove Jack's, Auckland, New Zealand) were purchased from a local brewery store Sovina (Porto, Portugal). Xanthohumol (XN) standard ≥99% purity was purchased from Extrasynthese (Z.I Lyon Nord, France). Stock standard solutions of 150 µg/mL in methanol were prepared and used for further dilution. Formic acid (98–100%), sodium acetate and ortho-phosphoric acid 85% was supplied by Merck (Darmstadt, Germany). Methanol and Acetonitrile were HPLC grade (≥ 99,9%). Bond Elut C18, 500 mg 3 mL columns were obtained from Agilent Technologies (Lake Forest, CA, US). Water was purified with a Milli-Q System (resistivity >18 MΩ cm) (Millipore, Bedford, MA, US).

7.2.2. Brewing process

An American-Style Pale Ale beer was produced from 5.5 kg of barley malt (5 kg of Best Pale Ale and 0.5 kg of Caramünch® III) in 20 L batches. According to a standard mashing procedure, the grain in 14 L of water was initially heated at 62°C during 60 min, followed by 10 min at 72°C. Afterwards, they were heated to 78°C for filtration and recirculation. At the beginning of wort boiling, 42 g of SUM hop pellets were added. Wort was boiled for 60 min at 100°C. The original gravity was evaluated by densitometry (EBC 8.2.2 method) and set to 1.052, and the aerated wort was fermented for 7 days at 20°C. Beer (three batches) was separated from yeast debris, gathered in an 80 L container and maturated for 21 days at 4°C. The final alcohol was evaluated by distillation (EBC. 9.2.1 method) and volume content reached 5.5 % (v/v).

7.2.3. Maturation and dry hopping techniques

Different dry-hopped beers were prepared in 0,5 L bottles from the base pale ale beer using 3 varieties of hops (harvested in 2017): a bitter high α-acids variety CHI; a hop with dual purpose, EKG; and an aroma variety with low content of α-acids, TET. Hops were added directly into the beers, three different hop concentrations (0.7; 1.4 and 2.8 g/ L) were

tested at 3 different points during the maturation (21, 10 and 5 days before the end of maturation). A control beer without dry-hopping was carried out by maturing the base matrix for 21 days without any addition of hops. All trials were done in triplicate. The three maturation times and concentrations to use were determined taking into account the most used dry hopping ratios in microbreweries (36).

7.2.4. Beer and hop samples preparation

After the maturation time, beers were centrifuged for 5 min at 1500 x *g* to separate the residual hop solids and stored at 4 °C until the analysis. A concentration step by solid phase extraction (SPE) using 500 mg Bond Elut C18 3 mL columns was required for α-acids and xanthohumol analysis in beer. Samples (20 mL) were acidified with 40 µL of 85% ortho-phosphoric acid. Conditioning of the SPE columns was carried out with 2 mL of methanol followed by 2 mL of ultra-pure water, acidified samples were then added to the SPE column, discarding the eluate. Treated columns were washed with 6 mL of acidified water (200 µL of 85% ortho-phosphoric acid in 100 mL of ultra-pure water) and 2 mL of an acidified solution of methanol and water (200 µL of 85% ortho-phosphoric acid in 50 mL of ultra-pure water and 50 mL of methanol). Elution of concentrates was made with 2 mL of acidified methanol (100 mL of 85 % ortho-phosphoric acid in 100 mL of methanol) (223). To quantify the percentage of α-acids and xanthohumol in the pellets of each variety of hops, extracts were prepared by maceration of 0.5 g of sample with 10 mL of methanol acidified with 1% formic acid (v/ v) for 30 minutes at room temperature, under stirring (30).

7.2.5. Analyses of α-acids and xanthohumol by reverse-phase liquid chromatography with ultra-violet diode array detection (RP-HPLC-UV-DAD)

HPLC analyses were performed according to the description of 3.2.7.

7.2.6. Statistical analyses

All dependent variables from analyzed hops and beers were tested for distribution of the residuals with Shapiro–Wilk's test. As normal distribution of the residuals was confirmed, a three-way analysis of variance (ANOVA) was applied using the α-acids and xanthohumol hop contents, the dose rates applied to beer, and the maturation time as the fixed variables.

Whenever statistical significances were found for interactions or main effects, Tukey´s or Dunnett's post-hoc tests were applied for mean comparison, depending on equal variances assumption or not. A two-way ANOVA was carried out in order to analyze α-acids and XN recovery (concentration in hop vs concentration in beer) throughout maturation time. Subsequently, nonlinear regression was used to study the relationships between beer parameters (Y-matrix) and hop parameter (X-matrix) in terms of prediction of Y-variables from X-variables. Random validation was also applied to identify relevant X-variables. Two and three-way ANOVA analyses were performed at 5% significance level, using Statistica, version 13 (Dell Inc, Tulsa, US). Nonlinear regression analyses were conducted with the XLSTAT® for Windows version 2016.02 (Addinsoft, Paris, France) at 5% significance level.

7.3. Results and Discussion

7.3.1. α-Acids and xanthohumol content in hops

The HPLC/ DAD method for XN and the different α-acids fractions (n-, co- and ad-humulones) was employed. Compounds identification and peak assignment was based on comparison of their UV-Vis spectrum data and retention times of standards. α-acids contents were determined as the sum of the three separated fractions. The obtained data from HPLC/ DAD quantification, in the three varieties of hops used for further assays, was for the total content of α-acids and XN were 12.5 ± 1.0 (w/w) and 0.50 ± 0.04 (w/w), respectively, in the case of CHI variety. For EKG were obtained 4.0 ± 0.2 (w/w) and 0.29 ± 0.02 for the total content of α-acids and XN, respectively. In TET variety were obtained 1.7 ± 0.1 (w/w) and 0.26 ± 0.01 for the total content of α-acids and XN, respectively.

The results show higher values in CHI than in the other tested hops, for the different compounds under study. CHI presented 12.5% of α-acids within the usual range (11.5 – 15.0 (w/w)) (224). EKG and TET presented values slightly lower than the usual range (4.5 – 6.5 (w/w) and 2.5 – 5.5 (w/w) (224), respectively), which can occur due to degradation of compounds during pellet storage (225, 226), that may occur even in the recommended shelf life. Concerning XN contents, the results ranged between 0.26 and 0.50 (w/w). Usually XN is present in hops in quantities between 0.1% and 1.0 % (w/w) (54), which is in accordance with the found values. However, its content highly depends on the variety, the year and the locale. For instance, in a study carried out with TET cultivated in Italy, the flowers presented 0.972 % (w/w) of XN (133).

7.3.2. Variation of α-acids and xanthohumol during brewing

The transfer of hop compounds to produce different beers involves an extraction process controlled by time and temperature, to achieve the selective transfer of these to beer or wort during brewing process (227). In this study the use of three points of time maturation for dry-hoping (21, 10 and 5 days) was an attempt to simulate the most used practices in dry-hopping by these brewer's, which may vary between short periods (228) up to longer periods (229). As expected, the beers dry-hopped with the varieties CHI, EKG and TET, at three concentration levels and three different times of maturation presented a wide range of amounts of the compounds under study. Results are summarized in Table 7.1 and, additionally, in Figure 7.1 for the convenience of visualization and interpretation. The α-acids ranged from not detectable, in control beers without dry-hopping, up to values over 20 mg/ L, much higher than the contents described for ordinary commercial beers (51, 219). A content of α-acids ranging from 0.0 to 5.3 mg/ L was described in non-dry-hopped and dry-hopped commercial lager beers (230), which are usually dry-hopped with low amounts of hops. In the present work, the highest amount for α-acids was achieved with the CHI variety at the dosage of 2.8 g/ L and 10 days of maturation, with a value of 22.5±0.3 mg/ L. The lowest concentration value was for 0.7 g/ L of TET and 5 days of dry-hopping, with 2.5±1.7 mg/ L.

Table 7.1. Mean values ± standard deviation (SD) for α-acids and xanthohumol for three different hops at three different dry-hopping concentrations throughout maturation time.

Compound Hops	[Hops] (g/ L)								
	0.7			1.4			2.8		
	5 days	10 days	21 days	5 days	10 days	21 days	5 days	10 days	21 days
Total α-acids									
CHI	13.7 ± 0.1aA	17.7 ± 0.0aB	10.1 ± 3.2aA	15.6 ± 0.3bA	19.6 ± 1.7bB	17.0 ± 2.9bA	16.4 ± 0.2bA	22.5 ± 0.3bB	19.3 ± 1.4bA
EKG	9.7 ± 0.2cdC	9.5 ± 1.2cdC	8.8 ± 0.1cdC	10.4 ± 0.5cC	10.0 ± 0.8cC	9.3 ± 1.2cC	12.2 ± 0.7aC	12.4 ± 1.8aC	13.2 ± 3.1aC
TET	2.5 ± 1.7eD	6.9 ± 0.2eE	7.5 ± 0.0eCE	4.2 ± 0.0eD	7.2 ± 0.5eE	7.6 ±0.2eCE	4.6 ± 0.1deD	7.4 ± 0.6deE	10.2 ± 1.1deCE
Xanthohumol									
CHI	0.62 ± 0.0aA	0.85 ± 0.0aB	0.56 ± 0.1aA	0.77 ± 0.1bA	1.03 ± 0.1bB	0.92 ± 0.3bA	0.91 ± 0.0cA	1.33 ± 0.0cB	1.24 ± 0.2cA
EKG	0.47 ± 0.0deCD	0.57 ± 0.1deAC	0.46 ± 0.1deCD	0.56 ± 0.1adCD	0.63 ± 0.1adAC	0.66 ± 0.1adCD	0.63 ± 0.1aCD	0.71 ± 0.1aAC	0.67 ± 0.1aCD
TET	0.25 ± 0.1eE	0.44 ± 0.0eDE	0.38 ± 0.0eD	0.30 ± 0.2efE	0.50 ± 0.1efDE	0.51 ±0.1efD	0.48 ± 0.0adfE	0.50 ± 0.1adfDE	0.61 ± 0.1adfD

Data expressed as mean ± standard deviation. Different lower case letters for each compound show statistically significant differences ($p < 0.05$) between means of hops at different concentrations. Different upper case letters for each compound show statistically significant differences ($p < 0.05$) between means of hops at different maturation time. The p values considered were obtained from three-way ANOVA analysis (Table 2). Means were compared by Dunnet's test, since homogeneity of variances was not confirmed by Levene's test ($p < 0.05$).

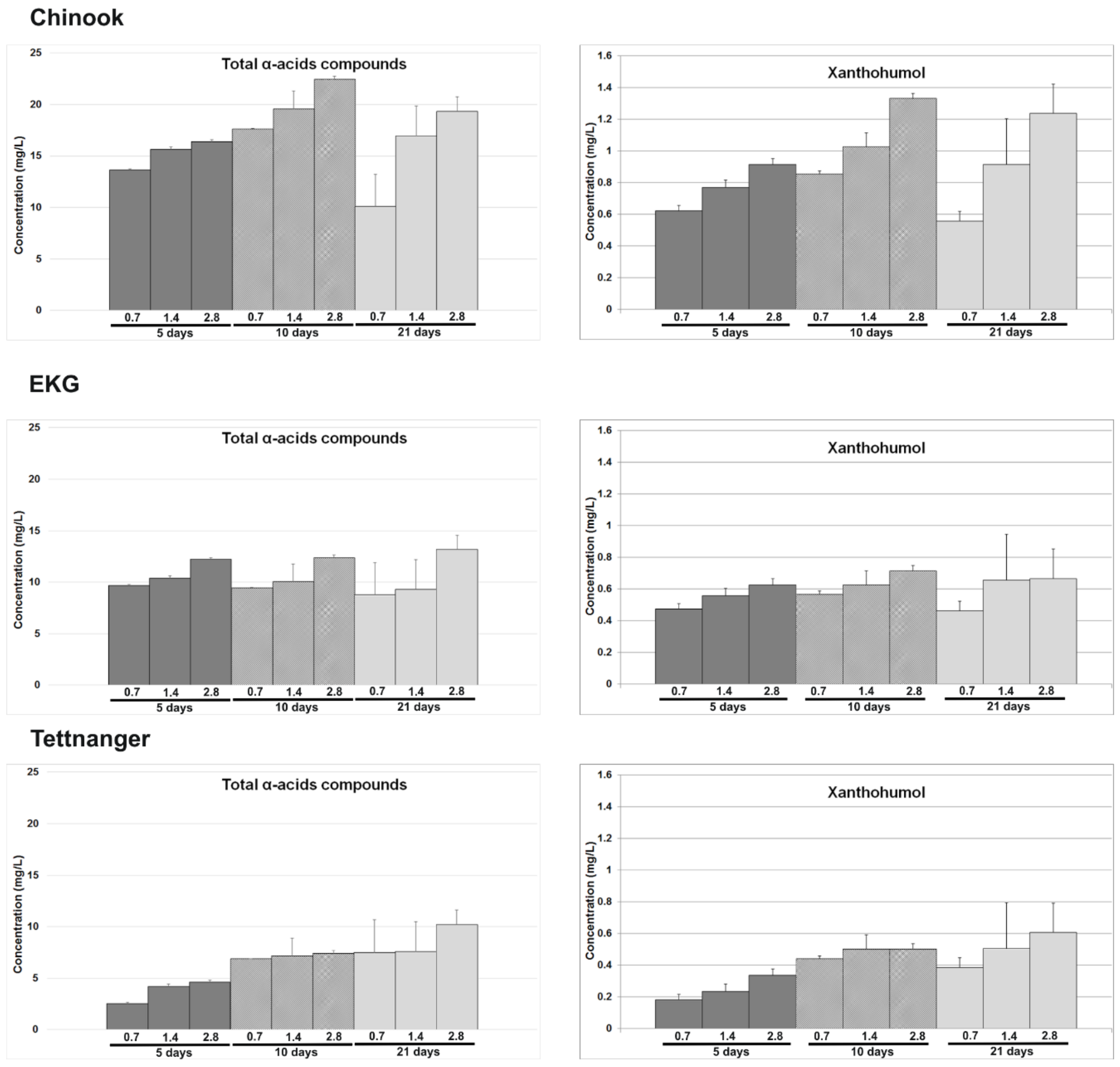


Fig. 7.1. Concentrations of α-acids and xanthohumol (mg/ L) in beers maturated with 0.7 g/ L, 1.4 g/ L and 2.8 g/ L of CHI, EKG and TET hops for 5; 10 and 21 days.

Similar behavior in terms of hop variety and dosage was noticed for XN, with 1.33±0.0 mg/ L as the highest value and 0.25±0.1 mg/ L as the lowest. These values were in agreement with those described in the literature (54, 231-233), although it has been reported that dry hopping techniques, with a combination of various types of hops at higher levels of addition, may increase XN contents in beer up to 10 mg/ L (50).

An overview of the obtained values, when comparing the same concentration and maturation time, indicates that beers prepared with CHI hops in general presented higher levels of α-acids and XN, whereas beers prepared with TET presented lower values. This profile of compounds transference from hop pellets to the beer matrix is in accordance with

the expected, since higher contents in the hops lead to higher levels of α-acids and XN in beer. Notwithstanding, the goal was to deepen the study on compounds transference to the beer, at the level of interaction of the different factors involved, in order to achieve a tool to the maximization of extraction in controlled conditions. For that purpose, a three-way ANOVA analysis was then selected to investigate the effects of three different factors (hop type, maturation time and hop concentration) as well as their interaction on α-acids and XN final concentrations, and the results are summarized in Table 7.2. The model was then validated by a regression analysis performed to fit the response functions; and the final model was obtained for α-acids and XN. Adequacy and significance of the quadratic model were evaluated by analysis of the variance (ANOVA) by means of Fisher's F-test.

Table 7.2. Results of three-way ANOVA analysis for total α-acids and xanthohumol in different hop concentration throughout maturation time.

Effect	df	Mean Squares	F	*p*	% variance
Total α-acids					
Hops (X1)	2	504.517	222.407	< 0.001	0.939
Maturation time (X2)	2	31.739	13.992	< 0.001	0.491
Hop concentration (X3)	2	55.911	24.647	< 0.001	0.630
X1 × X2	4	22.085	9.736	< 0.001	0.573
X1 × X3	4	7.405	3.264	0.025	0.310
X2 × X3	4	4.195	1.849	ns	0.203
X1 × X2 × X3	8	2.334	1.029	ns	0.221
Error	29	2.268			
Xanthohumol					
Hops (X1)	2	1.690	144.283	< 0.001	0.818
Maturation time (X2)	2	0.202	17.249	< 0.001	0.350
Hop concentration (X3)	2	0.540	46.144	< 0.001	0.591
X1 × X2	4	0.040	3.396	0.014	0.175
X1 × X3	4	0.086	7.331	< 0.001	0.314
X2 × X3	4	0.022	1.859	ns	0.104
X1 × X2 × X3	8	0.013	1.143	ns	0.125
Error	64	0.012			

ns, not significant. Significant *P*-values (<0.05). df, degrees of freedom. F, variance of group means divided by mean of the within group variance.

The models presented a good fitness to quadratic interaction with an F-test value of 23.73 and 20.33 for α-acids and XN, respectively, which implies that the models were significant for compounds. They also presented a good fitness for quadratic interaction, with R^2pred values of 0.9551 and 0.8920 for α-acids and XN, respectively, which are in agreement with the R^2adj value of 0.9148 and 0.8481 for α-acids and XN. Typical values indicating a good fit of models are $R^2 > 0.75$, here verified indicating the goodness of the model. Additionally, in order to evaluate the square root of the variance of the residuals, root mean square errors

(RMSE) were determined as 1.506 and 0.108 mg/ L for α-acids and XN, respectively. There was a statistical significance ($p < 0.05$) for all main effects under study. In the interactive effects, a statistical significance was observed for hops type and maturation time, and between hops type and hop concentration, but not for maturation time and hop concentration. Moreover, no statistical significance was observed between the three main effects (Table 7.2).

Afterwards, profiles for predicted values and desirability of different factors and their interactions are shown in Figure 7.2. Desirability was established as the highest amount of α-acids and XN, considering initial hop concentration and maturation time. It can be observed that the highest amount of α-acids was given by the following combination of factors: CHI hop, 10 days of maturation time, and 2.8 g/ L of hop concentration (Figure 7.2A). This observed decay in α-acids concentration in beer from 10 to 21 days is most probably related to the oxidation of α-acids naturally occurring during the assay and beer storage (230, 234). Concerning XN, the maximum desirability was achieved for a single point, except in maturation time where it was reached at 10 and 21 days; nevertheless, XN amounts were only within confidence interval of the analysis for 21 days. Thus, the combination for the highest amount of XN was CHI hops, 21 days of maturation time, and 2.8 g/ L of hop concentration (Figure 7.2B).

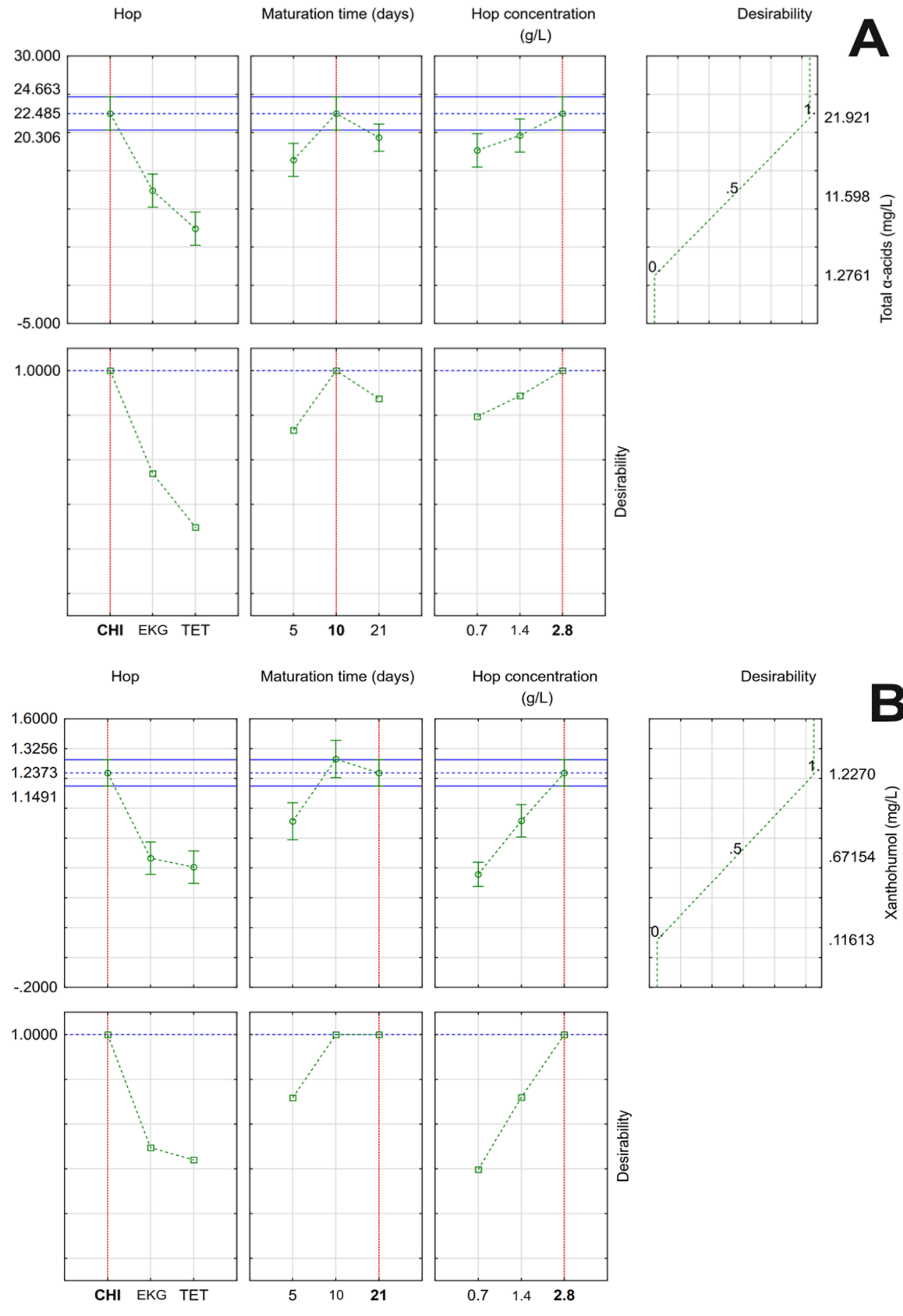


Fig. 7.2. Profiles for predicted values and desirability index in different hop addition rates throughout maturation time for α-acids (A) and xanthohumol (B).

7.3.3. Transfer rate from hops to beer

The results from section 7.3.2 suggest a tendency in which addition of higher amount of hops lead to higher content of α-acids and XN in the final beers. However, when the relation between α-acids and XN quantity in beers post-maturation, and the initial quantities of these compounds in added hops was analyzed (Table 7.3), it was observed that the highest transfer rate (extraction efficiency) was achieved at lower concentrations of dry-hopping (Figure 7.3 and Figure 7.4). Herein transfer rate was the ratio between final concentrations in beer versus initial concentration in added hops.

Table 7.3. Transfer rate percentage of the ratio between final concentration in beer versus initial concentration added by hops.

	Transfer rate (%)								
[Hops]	**0.7 (g/ L)**			**1.4 (g/ L)**			**2.8(g/ L)**		
Maturation time	**5 days**	**10 days**	**21 days**	**5 days**	**10 days**	**21 days**	**5 days**	**10 days**	**21 days**
Total α-acids									
CHI	15.68 ±1.18	20.28± 1.53	11.59 ± 3.25	8.98 ± 0.69	11.25 ± 1.14	9.74 ± 1.61	4.71 ± 0.36	6.45 ± 0.49	5.55 ± 0.54
EKG	34.39 ± 1.55	33.64 ± 3.58	31.15 ± 1.30	18.41 ± 1.01	17.85 ± 1.31	16.50 ± 1.85	10.85 ± 0.64	11.00 ± 1.30	11.69 ± 2.19
TET	21.40 ± 11.16	57.90 ± 2.75	62.97 ± 2.82	17.54 ± 0.71	30.12 ± 1.98	31.84 ± 1.45	9.67 ± 0.40	15.55 ± 1.20	21.40 ± 2.05
Xanthohumol									
CHI	17.90 ± 1.56	24.65 ± 1.87	16.08 ± 2.04	11.10 ± 1.00	14.80 ± 1.54	13.21 ± 3.89	6.59 ± 0.54	9.60 ± 0.73	8.93 ± 1.41
EKG	23.49 ± 1.76	28.22 ± 3.47	22.94 ± 4.14	13.81 ± 1.68	15.57 ± 1.61	16.29 ± 1.36	7.76 ± 0.75	8.86 ± 0.82	8.26 ± 1.64
TET	13.66 ± 4.99	24.08 ± 1.33	21.07 ± 1.23	8.11± 4.68	13.74 ± 1.29	13.86 ± 1.33	6.63 ± 0.27	6.90 ± 1.32	8.29 ± 1.13

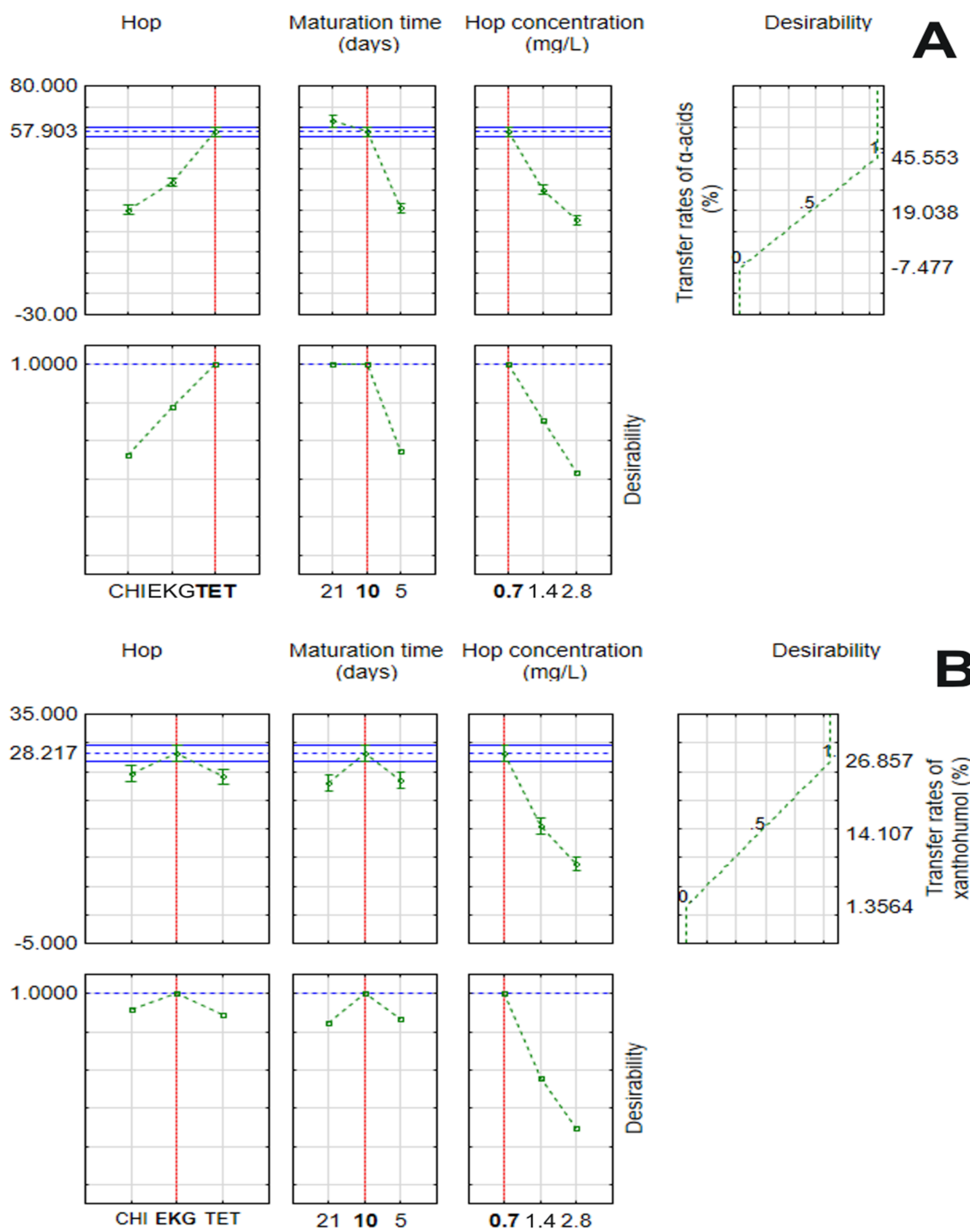


Fig. 7.3. Profiles for predicted values and desirability index of transfer rate from hops to beer for α-acids (A) and xanthohumol (B).

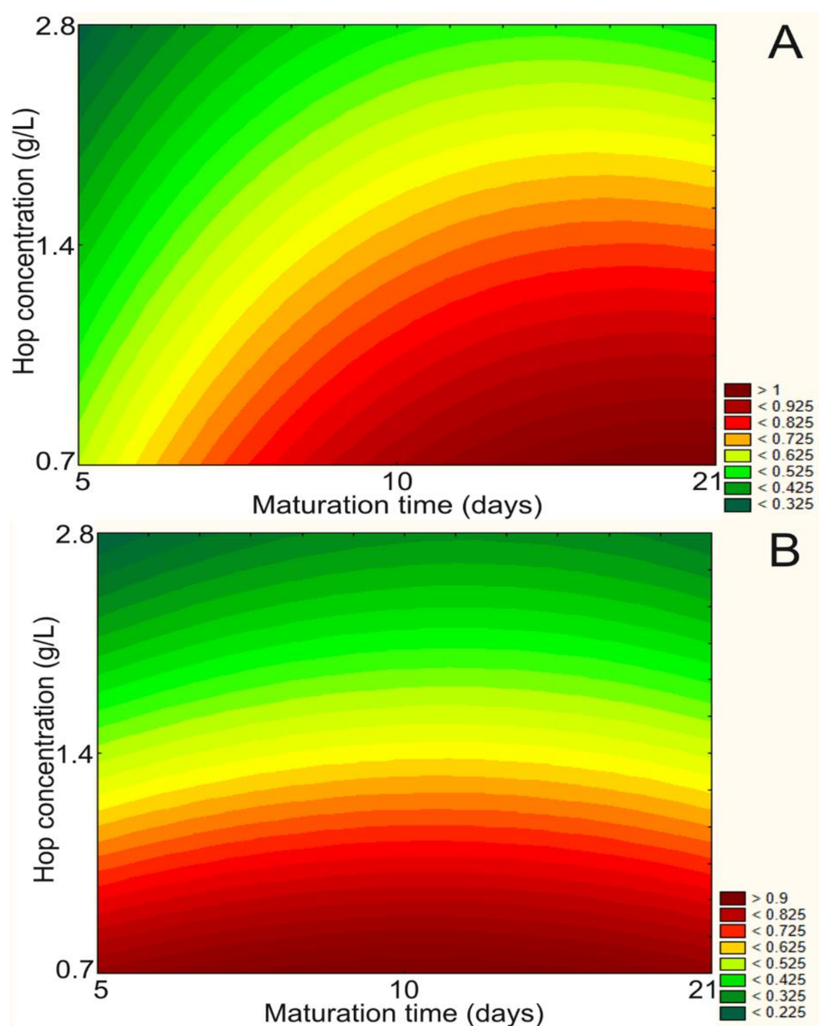


Fig. 7.4. Desirability surface contours by a method of quadratic fit (from 3 way ANOVA) in α-acids (A) and xanthohumol (B).

Again, a three-way ANOVA analysis was used to investigate the effects of hop used, maturation time and hop concentration, as well as the interactions on transfer rate of α-acids and XN to final beer. Table 7.4 summarizes the ANOVA results. Models were validated as described in section 7.3.2, presenting a good fitness to quadratic interaction with an F-test value of 148.81 and 84.82 for α-acids and XN, respectively. They also presented a good fitness for quadratic interaction, with R^2pred values of 0.9648 and 0.8996 for α-acids and XN, respectively, which are in agreement with the R^2adj value of 0.9584 and 0.8890 for α-acids and XN. Root-mean-square error (RMSE) was determined as 2.697 and 2.124 mg/ L for α-acids and XN, respectively.

Table 7.4. Results of three-way ANOVA analysis for total α-acids and xanthohumol transfer rate from hops to beer.

Effect	df	Mean Squares	F	*p*	% variance
α-acids					
Hop (A)	2	5098.16	701.074	<0.001	0.909
Maturation time (B)	2	843.69	116.020	<0.001	0.622
Hop concentration (C)	2	6188.86	851.062	<0.001	0.924
A × B	4	868.43	119.422	<0.001	0.772
A × C	4	547.00	75.221	<0.001	0.681
B × C	4	158.90	21.851	<0.001	0.383
A × B × C	8	192.39	26.457	<0.001	0.600
Error	141	7.27			
Xanthohumol					
Hop (A)	2	236.14	52.36	<0.001	0.299
Maturation time (B)	2	334.79	74.23	<0.001	0.376
Hop concentration (C)	2	3879.13	860.14	<0.001	0.875
A × B	4	48.67	10.79	<0.001	0.149
A × C	4	62.17	13.79	<0.001	0.183
B × C	4	95.54	21.19	<0.001	0.256
A × B × C	8	18.08	4.01	<0.001	0.115
Error	246	4.51			

df, degrees of freedom. F, variance of group means divided by mean of the within group variance

The extraction efficience of α-acids and XN demonstrated to be opposed to amount of hop addition. That is, highest percentages of compounds were extracted when lowest amount of hops were added. The highest transfer rate of α-acids (57.90 %) was observed for TET hop, 21 days of maturation, and 0.7 g/ L of hop concentration (Figure 7.3A). For XN the conditions for the highest transfer rate (28.22 %) was for EKG hop, 10 days of maturation time, and 0.7 g/ L of hop concentration (Figure 7.3B). Interestingly, the conditions for the optimal transfer rate of XN were quite similar to those observed for α-acids, concerning the maturation time and hops dosage, differing only in the variety of hops used. This behavior may be explained by the similar octanol/ water partition coefficient, since these molecules have a hydrophobic behavior.

Data concerning the extraction efficience of XN and α-acids to the beer is scarce in scientific literature however, in a study with XN enriched products by Wunderlich and collaborators, authors demonstrated that the recovery decreases with increasing XN dosages, which is in accordance with the present results (235).

7.3.4. Regression modeling for optimization of conditions

In order to obtain the maximum concentration of α-acids and XN in experimental conditions, the criteria for the variables under study were established after evaluation of previous results. Data from α-acids and XN, present in hops and in final beer, and

maturation time (Table 7.3) was analyzed using regression models. Equations (f(x)) with best fitting performance (higher R^2 and lower RMSE) were selected (6th degree polynomials). Afterwards, the first derivatives (f'(x)) were determined and f'(x) = 0 was found in order to establish maximum α-acids and XN achieved in beer and respective initial addition rates, and maturation time (Table 7.5). Although modeling could be carried out in individual hops, the goal was to try to find an extraction pattern, regardless of the hops used. If hops were considered individually, either 1st or 2nd degree polynomial equations would be enough to explain the relationship between variables and physical interpretation more evident. The use of a 6th degree polynomial equation when hops were considered collectively reflect differences inherent to each hop. Although physical interpretation is not as evident as with the ones resulting from hops individually, it provides valuable insights on the complexity of the extraction, an information that could not be obtained otherwise. With these results together with statistical analyses of regression models, it was possible to identify that the best extraction was reached in approximately 13.5 days of maturation. For α-acids, the optimal initial added concentration in beer is 147 mg/ L, which, calculating for the tested hops, represents 1.2 g/ L of CHI, or 3.7 g/ L of EKG, or 8.7 g/ L of TET. For XN, the optimal initial concentration is 13.9 mg/ L, which corresponds to 2.8 g/ L of CHI, or 4.8 g/ L of EKG, or 5.3 g/ L of TET.

Table 7.5. regression models (6th degree polynomial) and equations with best fitting performance of α-acids and xanthohumol.

Dependent Variable (Y)	Explanatory variables (X)	R^2/ RMSE	Maximum value
α-acids in beer (mg/ L)	Maturation time (**m**) α-acids content in hop (**a**)	0.926/ 2.732 (mg/ L)	**m** = 13.25 days **a** = 146.73 mg/ L
$Y = -11.61+1.59\mathbf{m}+0.98-6.00\times10^{-02}\mathbf{m}^2-3.19\times10^{-02}\mathbf{a}^2+5.51\times10^{-04}\mathbf{a}^3-4.70\times10^{-06}\mathbf{a}^4+1.82\times10^{-08}\mathbf{a}^5-2.45\times10^{-11}\mathbf{a}^6$			
Xanthohumol in beer (mg/ L)	Maturation time (**m**) xanthohumol content in hop (**n**)	0.937/ 0.148 (mg/ L)	**m** = 13.92 days **n** = 13.89 mg/ L
$Y = -14.33+0.13\mathbf{m}+19.58\mathbf{n}-4.6710^{-03}\mathbf{m}^2-10.48\mathbf{n}^2+2.78\mathbf{n}^3-0.38\mathbf{n}^4+2.58\times10^{-02}\mathbf{n}^5-6.66\times10^{-04}\mathbf{n}^6$			

Most of the published studies related to α-acids and XN transfer to beer are related to the influence of the type of malt in the extraction performance (235). This study, however, was performed only with pale malts, used on most of the beers in which the dry-hopping technique is applied, as the popular India Pale Ales (IPA), in order to assess the effect of the other variables involved.

The equations obtained in this study can be applied in the calculation of the minimum amount of hops to add in the dry-hopping process in order to reach a defined concentration of α-acids or XN in the final beer. These can be helpful to the beer industry in mainly to aspects, either to use the minimum amount of hops to reach a desirable concentrations and to control (predict) the amount of α-acids and XN in the final product to increase its bioactivity.

7.4. Conclusions

A general tendency was observed indicating a positive correlation between the quantity of hops added and the levels of α-acids and XN present in beers, as expected. The concentration of α-acids in beers post-maturation reached values higher than 20 mg/ L, for a combination of CHI hops, 10 days of maturation time, and 2.8 g/ L of hop concentration, which was higher than level found in literature for commercial beers. For XN, a similar behavior was verified concerning the type of hops, maturation time and concentrations. The highest transfer rate was observed in the beers that presented the lowest initial content for the compounds under study. The optimal concentrations of compounds to be added to beer in order to reach the maximum transfer rate (maximum value with the minimal amount of hops added) were determined by analyzing the results from regression models and equations, being achieved at approximately 13.5 days of maturation, with the amount of hops corresponding to the added concentrations of α-acids and XN of 147 and 13.9 mg/ L, respectively. Equations (f(x)) with best fitting performance were selected (6th degree polynomials) for prediction of α-acids and XN concentrations in beer. These findings can be of high relevance, as it provides a better knowledge of the extraction behavior of important bitter and bioactive compounds present in hops. In the present scenario of steady increment in the use of dry-hopping techniques by both the brewing industry and small producers, as well as the consumer’s demand for higher bioactivity in foods and beverages, the predictability of the bioactivities α-acids and XN concentration in the final product will be highly valued.

CHAPTER 8

Antiproliferative effect of beer and hop compounds against human colorectal adenocarcinoma caco-2 cells

8.1. Introduction

The increase of hop utilization promotes the production of beers with higher content in bioactive compounds, which is in line with the worldwide trend to produce foods and beverages with increased bioactivity and beneficial health properties.

The evaluation of beer as a source of of xanthohumol (XN), isoxanthohumol (IXN), α and β-acids (ABA) and iso-α-acids (IAA) is pertinent since, at least for hop bitter acids, there are studies referring the fast and efficient intestinal transport across colorectal adenocarcinoma Caco-2 cells (236). However, hop compounds activity information regarding Caco-2 cell response is missing. Therefore, the major goal of the present work was the evaluation of antiproliferative properties XN, IXN, ABA and IAA, both as pure compounds and as part of the beer matrix, using the Caco-2 cells as a model. This work is the first report on the bioactivity of hop compounds on Caco-2 cells using both the isolated compounds and the whole beer matrix exposed to cells *in vitro*.

8.2. Materials and methods

8.2.1. Chemicals and materials

The beer ingredients, malts pilsen Bestmalz (Heidelberg, Germany) and yeast Lager Brewferm (Beverlo, Belgium) were purchased from a local homebrew store. Xanthohumol (XN) and Isoxanthohumol (IXN) standards were purchased from Extrasynthese (Z.I Lyon Nord, France). International Calibration Extract (ICE-3), containing a mixture with 44.64% of α-acids (humulone, cohumulone and adhumulone) and 24.28% of β-acids (lupulone, colupulone and adlupulone) (ABA), and International Calibration Standards DCHA-ISO, ICS-I3, containing 62.3% of total iso-α-acids (IAA), were from Labor Veritas (Zurich, Switzerland). The human cell line Caco-2 (passages 18–35) with its origin in a human colorectal adenocarcinoma was made available by the "Molecular Physical-Chemistry" research Group of the University of Coimbra, Portugal. High glucose Dulbecco's modified Eagle's medium (DMEM), Minimum essential medium non-essential amino acids (MEM NEAA) 100×, GlutaMAX™ 100×, fetal bovine serum (FBS), 0.25% Trypsin-EDTA solution and Penicillin/Streptomycin 100× solution (10,000 Units mL–1/10,000 µg/ mL) were all purchased from Gibco/Life Technologies corporation (Paisley, United Kingdom). MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) and dimethyl sulfoxide

(DMSO) were from Sigma-Aldrich Corp. (St. Louis, MO, US). All flasks and 96 well plates for cell assays were obtained at Corning (Ma, US).

8.2.2. Brewing process and sample preparation

Non-hopped Beer (NHB) was produced from 180 g of barley malt (15.0% Pilsen type) being the mashing schedule carried out for 60 min. at 62 °C and 15 min. at 78 °C. Wort was subsequently boiled for 60 min. at 100 °C, fermented for 7 days at 20 °C and maturated for 21 days at 4 °C, the original gravity was 1.027 and final gravity 1.005, calculating about 3% alcohol by volume. After maturation beer was frozen at - 80 °C and then lyophilized for ethanol removal. A total of 1,200 mL of beer was produced, without the addition of hops in any stage.

8.2.3. Cell culture and cytotoxicity tests

Caco-2 cells were routinely maintained in DMEM supplemented with 10% fetal bovine serum, 1% non-essential amino acids solution, 100 Units/ mL penicillin, 100 µg mL streptomycin and 1% GlutaMAX™ solution culture media (CM). Cells were incubated at 37 °C and 5% CO2. Medium was changed every two days and cells passed at 70% confluence. For cytotoxicity assays, cells were used between passages 21 and 35. Contamination with Mycoplasma was assessed by PCR every four weeks using protocol previously described (237).

An MTT assay was carried out to test cytotoxicity in Caco-2 cells for the exposition period of 48 h. Cells were seeded in 96 well plates (TPP, Trasadingen, Switzerland) at a density of 1.25 × 104 cells/ mL in 200 µl DMEM per well. After cell adhesion for 12 h the medium was replaced with the test solutions containing serial dilutions of the compounds or the beer matrix and incubated at 37 °C with 5% CO2 for the exposition period. Solutions of the compounds were prepared 1000 times concentrated in DMSO, for XN, IXN and ABA, and methanol for IAA. Concentration solutions were then diluted with CM, or a solution of the lyophilized beer reconstituted in CM, in order to obtain the final seven points serial dilutions from 0.313 to 20.000 mg/ L for XN and IXN; 1.25 to 80.00 mg/ L for ABA and 0.164 to 100.000 mg/ L for of IAA. A 2-fold serial dilution of NHB in CM was also prepared from 1.25 to 100% beer matrix. Vehicle controls covering all the concentrations of the solvents were performed. All standard compounds and solutions prepared were protected from light by the use of amber vials and the protection of plastic microtubes, as well as cell culture plates, with aluminum foil. Plate readings were performed in a Spectrostar Nano plate reader (Ortenberg, Germany).

8.2.4. Statistical analysis

All measurements were taken in quadruplicate in at least two independent experiments, and data were reported as mean ± standard deviation. One-way ANOVA followed by Dunnett's multiple comparisons test against controls was performed using GraphPad Prism version 7.00 for Windows, GraphPad Software, La Jolla California US, www.graphpad.com. IC50 values were determined by curve fitting using the equation log (inhibitor) vs. response – Variable slope (four parameters). Statistical significance was set for $P < 0.05$, where the null hypothesis claims that there is no difference between the compared samples.

8.3. Results and discussion

8.3.1. Antiproliferative activities of in Caco-2 cells

The antiproliferative activities of XN, IXN, ABA and IAA were tested in different ranges of concentration, 0.313–20 μg/ mL for XN and IXN, 1.25–80 for ABA and 5.85–100 μg/ mL for IAA. Doses were chosen taking into consideration the average amounts occurring in different types of beers, and the possibility of applying different hopping practices that can increase the range of bitter compounds. According to the literature IXN content of commercial beers range from 0.03 μg/mL to values slightly higher than 4 μg/ mL (105, 219), whereas XN usually does not exceed 1 μg/ mL. Dry hopping techniques may increase its content up to 10 μg/ mL of XN (50, 238). ABA content range between 0.4 and about 5 μg/ mL, whilst IAA achieved higher dosages, varying from 1.4 μg/ mL to about 40 μg/ mL (49, 105, 219). Overall beers contain equivalent levels of XN and IXN, thus similar concentration range was assayed, whereas the presence of higher amounts of ABA and IAA justify the assays at higher concentrations.

Figure 8.1 presents the results obtained by exposing Caco-2 cells to XN solutions, in complete culture medium, during 48 h, where XN showed no significant inhibitory effects in Caco-2 cells proliferation for the tested concentrations.

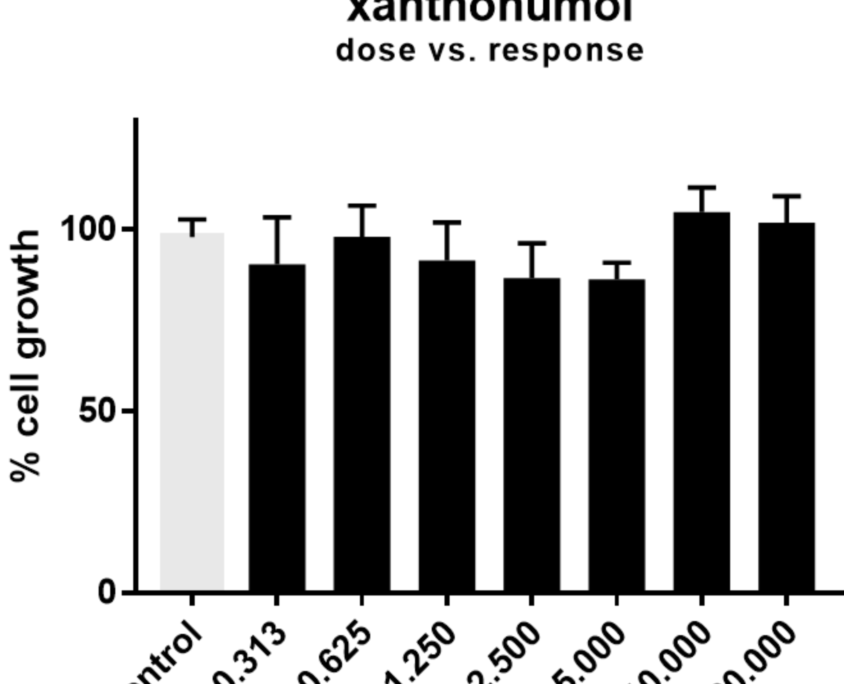


Figure 8.1. Effect of xanthohumol solutions in CM (0.313-20 µg/ mL) on Caco-2 cell growth after 48 hours exposure. All values are expressed as percentage to control. All means were compared and are not significant at $P < 0.05$.

A few previous studies, concerning colon cancer cell lines HT-29, HCT-116 and SW160 showed antiproliferative activity of XN at relatively low IC50 values from 1.2 to 2.5 µM (0.43–0.88 µg/ mL) (127, 239), whilst some other studies denoted inhibitory effects only at higher concentrations starting from 10 to 100 µM (3.54–35.4 µg/ mL) (240, 241). Henley et al. (2014) exposed HCT-116 cells to XN at the concentration of 3.5 µg/ mL and found significant inhibitory effect on cells proliferation and in apoptosis increase, however at values lower than 50% inhibition. Despite the relatively low values of IC50 found in some of the referred works, others report XN as less active (<50% inhibition) even at the higher concentration of 35.4 µg/ mL (241) as referred. These last values are more in line with the present data since we did not find any activity at the concentration of 20 µg/ mL. This lack of inhibitory effect specifically on colon cancer Caco-2 cell growth can also be inferred from the work of Pang et al. (2007). These authors investigated the transport of XN at a concentration of 50 µM, in Caco-2 monolayers, to understand the bioavailability of xanthohumol, and observed no decrease in the monolayer integrity after 2 h of incubation which is a good indicator of the cells resistance to the compound (242), despite the lower

incubation time used and the fact that Caco-2 cells are differentiated to enterocytes in transport experiments. Notwithstanding, differentiated Caco-2 cells can still express the colonocyte phenotype (243).

Contrarily to XN, results indicated that IXN showed significant inhibitory effect (ca. 25%) in Caco-2 cells proliferation at the highest concentrations tested of 10 and 20 μg/ mL. Figure 8.2 presents the results obtained by exposing Caco-2 cells to IXN, in the same conditions of XN.

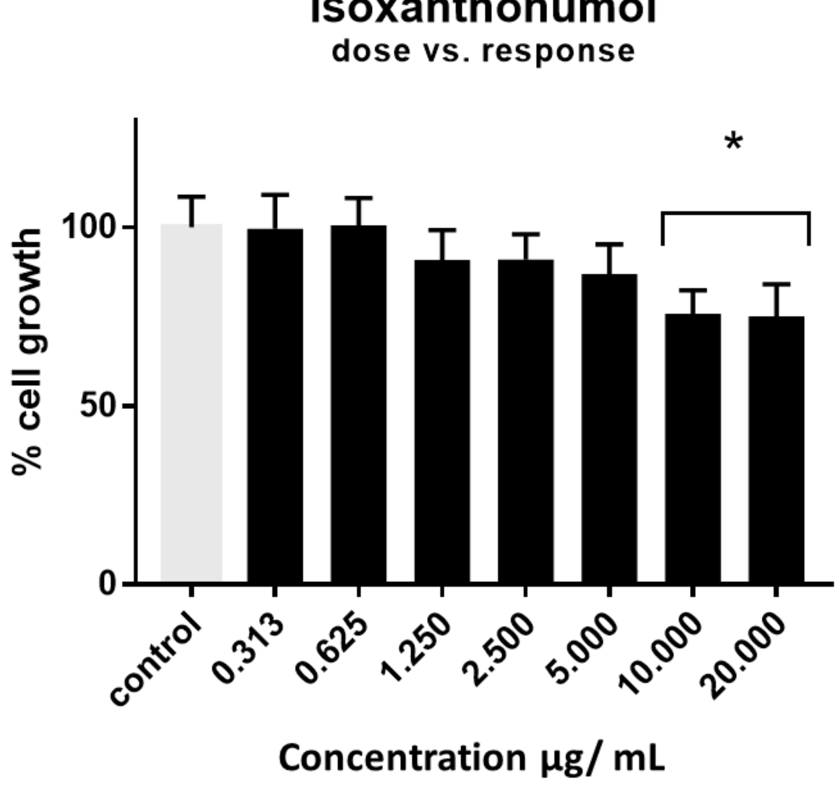


Figure 8.2. Effect of isoxanthohumol on Caco-2 cell growth after 48 hours exposure. All values are expressed as percentage to control. *significant at $P < 0.05$.

The effect of IXN on the proliferation of other colon cancer cells indicate that IXN exerted less inhibitory effects than XN presenting IC50 values of 16.9 μM and 37.3 μM (5.99 and 13.22 μg/ mL) for HT-29 and SW160 lines, respectively, where values for XN were 0.43 and 0.88 μg/ mL (127). Concerning HCT-116 cells exposed to IXN at the concentration of 2 ng/ mL and to a mixture of XN: IXN (12.69:1 ng/ mL) no significant effect on cells proliferation

neither in apoptosis increase was found (50). Notwithstanding, and reflecting the same behavior found for XN, some other studies (241) denoted inhibitory effects less than 50% only at the higher concentrations of 100 µM (35.4 µg/ mL). These last values are in accordance with the present results where about 25% growth inhibition was noted at concentrations higher than 10 µg/ mL, despite the fact that this study was performed in a different cell line. The mechanism of action suggested for XN and IXN concerning the activity in colon cancer cells is related to the inhibition of DNA synthesis (241) and apoptosis affecting several pathways (50, 127, 239, 240).

XN and IXN antiproliferative activity has also been studied in several other cell lines from different tissues (Table 8.1). Most of the times the substances were studied simultaneously permitting the direct comparison of their potency. The compounds effect in breast cancer cells MCF-7 (241, 244) and MDA-MB-435 (245) were described with IC50 values ranging from 3.47 to 25 µM. Gerhauser et al. (2002) proposed the inhibition of DNA polymerase as one of the mechanisms of activity in MDA-MB-435 cells.

Table 8.1. Published works concerning the antiproliferative effects of xanthohumol, isoxanthohumol, α and β-acids rich extracts on different human cell lines. #IC$_{50}$ values are indicated with bold characters. **commercial calibrated extracts.*

Compound	Cell line	Active dose#	Reference
Xanthohumol	Estrogen-dependent breast cancer (MCF-7)	3.47 µM	Miranda et al., 1999
		50 µM	Monteiro et al., 2008
	Colon cancer (HT-29)	100 µM	Miranda et al., 1999
		10 µM	Hadjiolov and Frank, 2009
		1.2 µM	Hudcová et al., 2014
	Ovarian cancer (A-2780)	0.52 µM	Miranda et al., 2000
	Mammary adenocarcinoma (MDA-MB-435)	25.0 µM	Gerhauser et al., 2002
	Promyelocytic leukemia (HL-60)	3.7 µM	
	Colon cancer (HCT-116)	2.3 µM	Pan et al., 2005
		10 µM	Hadjiolov and Frank, 2009
		3.5 µg/ mL	Henley et al., 2014
	Fibrosarcoma (HT-1080)	3 µM	Goto et al., 2005
	Prostate cancer cells (PC-3)	13.2 µM	Delmulle et al., 2006; 2008
	Prostate cancer cells (DU145)	12.3 µM	
	B-chronic lymphocytic leukemia (B-CLL)	24.4 µM	Lust et al., 2005
	Hormone-sensitive LNCaP (AR+)	20 to 40 µM	Deeb et al., 2010
	Hormone refractory PC-3 (AR–)	20 to 40 µM	
	DU145 (AR–)	20 to 40 µM	
	Prostate cancer (C4-2 derived from LNCaP)	20 to 40 µM	
	hepatocellular carcinoma (HepG2)	25 µM	Dorn et al., 2010
	hepatocellular carcinoma (Huh7)	25 µM	
	Acute lymphocytic leukemia (Nalm-6)	2.5 to 10 µM	
	Acute lymphocytic leukemia (697)	2.5 to 10 µM	
	Acute lymphocytic leukemia (RS 4;11)	2.5 to 10 µM	
	Acute lymphocytic leukemia (ALL-PO)	2.5 to 10 µM	
	Human colon adenocarcinoma (SW620)	2.5 µM	Hudcová et al., 2014
	Pancreas PANC-1	10–50 µM	Jiang et al., 2015
	Pancreas BxPC-3	10–50 µM	
	Larynx squamous cancer (RK33)	1 µM	Sławinska-Brych et al., 2015
	Larynx squamous cancer (RK45)	5 µM	
	Human skin fibroblasts (HSF) non-malignant	15 µM	
Isoxanthohumol	Human breast cancer (MCF-7)	4.69 µM	Miranda et al., 1999
	Ovarian cancer (A-2780)	18.0 µM	
	Colon cancer (HT-29)	100 µM	
		16.9 µM/ dm3	Hudcová et al., 2014
	Mammary adenocarcinoma (MDA-MB-435)	100 µM	Gerhauser et al., 2002
	Promyelocytic leucemia (HL-60)	DNS	
	Prostate cancer (PC-3)	45.2 µM	Delmulle et al., 2006; 2008
	Prostate cancer (DU145)	47.4 µM	
	Colon cancer (HCT-116)	2 ng/ mL	Henley et al., 2014
	Colon adenocarcinoma (SW620)	37.3 µM/ dm3	Hudcová et al., 2014

Table 8.1. Published works concerning the antiproliferative effects of xanthohumol, isoxanthohumol, α and β-acids rich extracts on different human cell lines. #IC_{50} values are indicated with bold characters. **commercial calibrated extracts* (continued).

Compound	Cell line	Active dose#	Reference
α-β-acids* 49.4% α-acids 24.9% β-acids	Promyelocytic cells leukemia (HL-60)	8.67 μg/ mL	Chen et al., 2004
	Histiocytic cells leukemia (U937)	58.87 μg/ mL	
α-acids rich* 57.2% α-acids 18.3% β-acids	Hepatocellular carcinoma (HepG2)	25 μg/ mL	Saugspier et al., 2012
	Hepatocellular carcinoma (PLC)		
	Hepatocellular carcinoma (Hep3B)		
β-acids rich* 13.0% α-acids 51.9% β-acids	Hepatocellular carcinoma (HepG2)	15 μg/ mL	Saugspier et al., 2012
	Hepatocellular carcinoma (PLC)		
	Hepatocellular carcinoma (Hep3B)		

Ovarian cancer cells A2780 were also studied (241) resulting in IC50 values of 0.52 to 18 μM as well as hormone-sensitive human prostatic cancer cell lines LNCaP (AR+), hormone refractory PC-3 (AR−) and DU145, and hormone-refractory (AR+) C4-2 (246-248). In prostate cell the inhibitory effect was attributed to the apoptosis-inducing effect (248).

Leukemia cells (HL-60, B-CLL, Nalm-6, 697, RS-4, RS-11 and ALL-PO) were also exposed to XN and IXN in several studies (245, 249, 250). In this type of cells the proposed mechanisms of action were the induction of apoptosis mainly by activation of caspases 8 and 9. In hepatocellular carcinomas HepG2 and Huh7, XN inhibited proliferation by decreasing cell migration and interleukin-8 expression (249).

Studies with pancreas tumor cell lines PAC1 and BxPC-3, XN showed antiproliferative activity inducing apoptosis by down regulating of the STAT3 signaling cell pathway (251).

Larynx squamous cancer cells RK33 and RK45 (252) were sensitive to XN at concentrations from 1 to 5 μM by inducing proapoptotic effectors Bcl-2, caspase-8 and caspase-9. These authors also verified that XN presented toxicity in non-malignant skin fibroblasts only at 15 μM. HT-1080 human fibrosarcoma cells were also studied concerning its response to XN at 3 μM which significantly suppressed cell proliferation however only in hypoxic conditions (253).

The other two classes of compounds study herein were α and β-acids (ABA), or humulones and lupulones, and iso-α-acids (IAA) or isohumulones, both in the form of a mixture (i.e. extract). Figure 8.3 presents the results obtained by exposing Caco-2 cells to solutions of the two mixtures dissolved in complete culture medium during 48 h period. Dose-dependent responses were obtained for both ABA and IAA and IC50 values were calculated as 16.16 ± 3.11 μg/ mL (ABA) and 50,61 ± 13.08 μg/ mL (IAA).

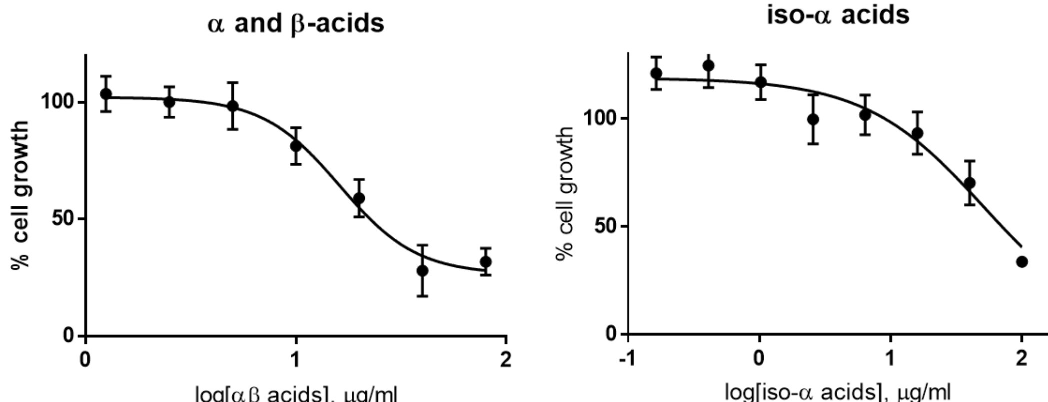


Figure 8.3. Log-dose vs. response curve for α and β-acids and iso-α-acids extracts at concentrations from 1.25 to 80.00 μg/ mL and 5.85 to 100.00 μg/ mL on Caco-2 cell growth after 48 hours exposure. For iso-α acids graphic was constraint at the bottom as equal to 0.

Alpha and β-acids were only scarcely studied concerning their bioactivity in cancer cells (Table 8.1). Chen and Lin (2004) studied the effects of ABA extracts in promyelocytic (HL-60) and Histiocytic (U937) leukemia cells reporting IC50 values of 8.67 and 58.87 μg/ mL respectively (254). In the present work we found the IC50 value of 16.16 ± 3.11 μg/mL for ABA extracts in Caco-2 cells which demonstrates a similar antiproliferative activity in this type of cells and reinforces the potential pharmacological use of these substances. Saugspier et al. (2012) studied the effect of α-acids rich extracts and β-acids rich extracts (containing both α-acids and β-acids however with enriched amounts in α-acids and β-acids, respectively) in hepatocellular carcinoma cells HepG2, PLC and Hep3B. Authors exposed the cells to solutions in a shorter period (24 h) which didn't inhibit the proliferation below 50% thus IC50 values were not determined (255). Notwithstanding values of about 25% inhibition were reported at 25 μg/ mL of α-acids and 15 μg/ mL of β-acids for the three types of hepatocellular carcinomas studied. As referred, we have found IC50 value of 16.16 ± 3.11 μg/ mL for Caco-2 cells, using a mixture of both bitter acids, however one cannot completely parallel these values as exposure time was the double in the present work. The pure constituent compounds of bitter acids humulone, isohumulone, lupulone and colupulone as well as extracts containing only α-acids or β-acids, have been studied during the last 20 years concerning their activity in leukemia, prostate, colon, breast and lung cancer cells (256-262). Few mechanisms of action were proposed, mainly based on apoptosis via extrinsic pathways either via caspases activation or involving the Fas or TNF paths. Despite the paramount findings achieved in the study of individual compounds in the last years, in the present work the goal was also to study the beer bioactivity in a "shotgun" approach as

opposed to the "silver bullet" method of studying the substances individually. This approach is based on the idea that a whole or partially purified extract of a plant offers advantages over a single isolated ingredient due to possible synergistic effects (263). One of the objectives herein was thus to study hop compounds as they appear in beer, which is nonetheless a hops and barley aqueous extract.

8.3.2. Influence of hops compounds on beer antiproliferative activity

In order to better understand the meaningfulness that hops compounds have over the beer antiproliferative activity we exposed Caco-2 cells to No Hopped Beer (NHB) solutions (containing all beers components except the ones originated form hops) and NHB spiked with known concentrations with the compounds of hops whose antiproliferative action (IXN, ABA, IAA) in cells had been previously verified. Figure 8.4 presents the results obtained by exposing Caco-2 cells to successive dilutions of lyophilized NHB in complete culture medium.

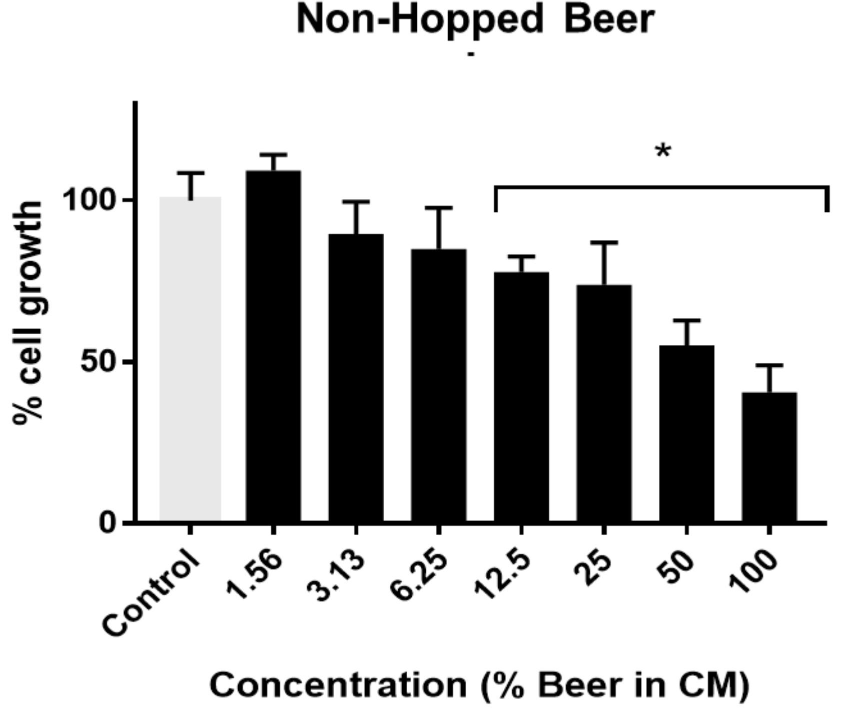


Figure 8.4. Effect of non-hopped beer (NHB) dilutions on Caco-2 cell growth after 48 hours exposure. All values are expressed as percentage to control growth only in culture medium. *significant at P < 0.001.

NHB solutions showed an antiproliferative activity from the concentration of 3.13% with a dose-dependent response profile until the maximum of 60% inhibition corresponding to

100% beer matrix in cell culture medium. The maximum antiproliferative activity (about 60%) obtained with lyophilized beer reconstituted in the same volume of culture medium (100% beer matrix), was similar to that achieved for 40 µg/ mL ABA and for 100 µg/ mL IAA of pure compounds, which indicates that the matrix itself contains potent bioactive substances most probably related to phenolic compounds from the malt. Moderate consumption of beers is not associated with epidemiological increase of cancer risk, instead having a great potential of cancer protection due the anticarcinogenic activity of its compounds (264-266). There are a large number of studies with beer compounds effect in cell proliferation however the effect of the whole beer in relation to in vitro cancer cell proliferation was only reported by Henley et al. (2014). According to this study, about 65% inhibition in HCT colon cancer cells was found for some India Pale Ale beers, equally diluted in culture medium at the concentration of about 3%. Despite the fact that different cell lines were used, Henley found higher activity in their experiences (for the same 3% concentration in beer we determined 10% inhibition against the 65% of the referred work) however it should be noticed that we used a beer matrix with no hop added thus with lower potential bioactivity (50).

In order to verify if the antiproliferative activity of the beer matrix can play a synergistic role in the antiproliferative activity of the pure compounds IXN, ABB and IAA, we performed another experience in which these compounds were spiked in 100% NHB beer matrix, at the same concentrations used for the previous assays with the pure molecules. The concentration of beer matrix was used at the maximum inhibition previously verified to assure that higher antiproliferative activity likely verified was due to the compound interaction with the matrix, i.e. to verify if the activity of the spiked beer matrix could reach higher values than the matrix alone or the isolated compounds. In these conditions an increase in the inhibition capacity would indicate that synergistic effects occur in the presence the whole beer matrix. Results obtained are expressed in Figure 8.5.

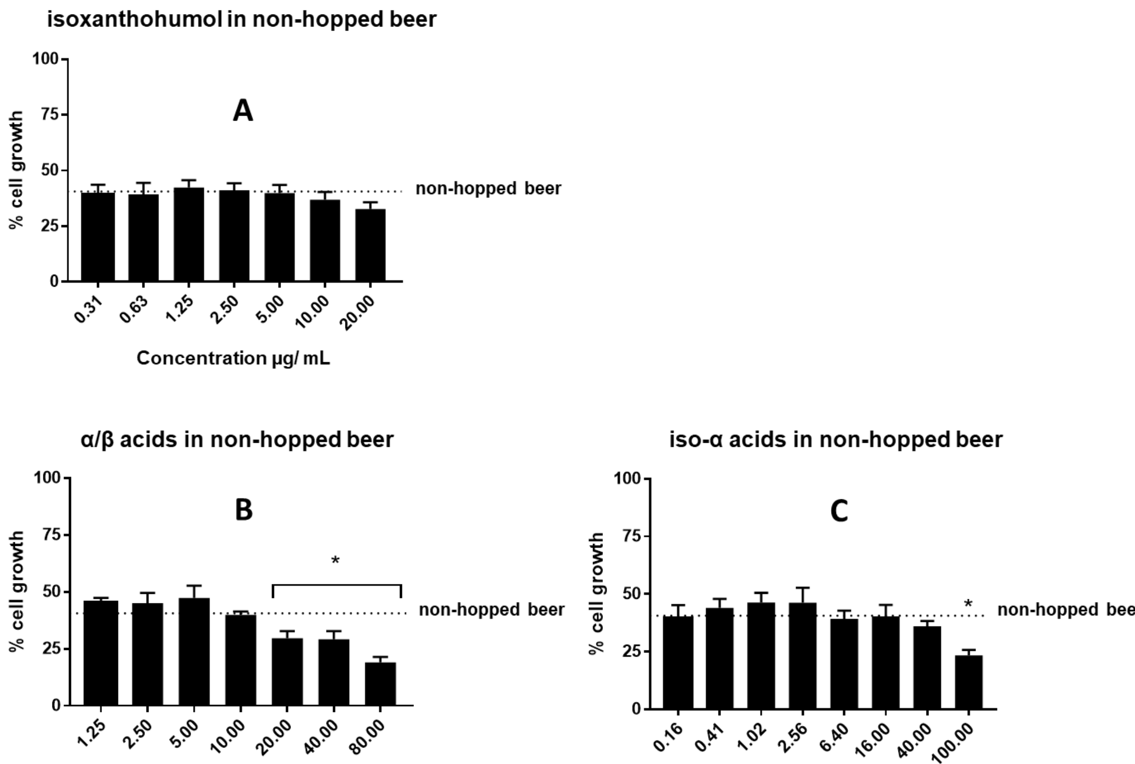


Figure 8.5. Effect of isoxhanthohumol, α/β acids and iso-α acids spiked in 100% beer matrix on Caco-2 cell growth after 48 hours exposure. All values are expressed as percentage to control. *significant at $P < 0.001$.

As expected, all experience replicates resulted in at least 50–60% inhibition corresponding the base effect of the beer matrix. The addition of IXN in concentrations ranging from 0.31 to 20 µg/ mL did not resulted in significant effect in the base activity of the beer (Figure 8.5A). Contrarily both ABA and IAA resulted in a significative increment in the antiproliferative activity at the highest concentrations. The addition of ABA to the beer matrix promoted the most notorious increase in activity at concentration from 20 to 80 µg/ml corresponding to 70.3 to 81.0% proliferation inhibition (Figure 8.5B). At the maximum concentrations of 80 µg/ mL ABA increased the beer antiproliferative activity by about 36%. Concerning IAA (Figure 8.5C), a significate increment in activity was noticed at the highest concentration of 100 µg/ mL leading to a reduction in the cells growth from about 40% to 23% corresponding to an effect equivalent to the ABA at 80 µg/ mL. The synergism of antiproliferative effects occurring in beer was previously reported by Henley et al. (2014). In their study, focused in XN and IXN, researchers showed that beer matrices had greater levels of suppression of cell proliferation and elevated apoptosis when compared with isolated components at the same concentration founded on commercial beers, indicating that the combination of the molecules tested with others inherent beer

compounds was more potent than isolated XN and IX in suppressing cancer cell proliferation (50). This finding can be of high relevance in the evaluation of beer as a potential bioactive food matrix, since it contains mixtures of ABA, IAA and IXN, on variable concentrations, depending on the technology used for production and the beer style itself.

8.4. Conclusions

The most relevant bioactive substances from hop were tested for their antiproliferative activity in colon adenocarcinoma Caco-2 cells. Xanthohumol did not show antiproliferative activity in the referred cells despite it presented high activity in other previous studies performed in HT-29, HCT-116 and SW160 cancer cells. Isoxanthohumol however was able to inhibit proliferation by about 25% at 10–20 μg/ mL when in pure solution in CM and increase by 7 and 13% the activity of the matrix beer when spiked at the same concentrations. The bitter acids, α and β-acids and iso-α-acids, showed to be the most active in Caco-2 cells with IC50 values of 16.16 ± 3.11 μg/ mL and 50.61 ± 13.08 μg/ mL, respectively. A synergist effect with the beer matrix was also noticed most pronounced for α and β-acids which increased the activity of the beer matrix by 36% at 80 μg/ mL but also for iso-α-acids with an increase of 23% in the activity of the matrix at 100 μg/ mL. The antiproliferative effects exerted over Caco-2 cells can thus be attributed to the beer base matrix components, however much leveraged by ABA and IAA contents. This synergistic effect is scarcely reported in literature and was herein studied concerning the three main bioactive single components of beer in what respects Caco-2 proliferation inhibition.

Since the brewing process and hops content of beer determine the beer richness in these active substances both can have an impact in the overall bioactivity of the drink as well as in its putative cancer preventive potential.

PART V

Overall conclusions and prospects

Overall conclusions and prospects

Nowadays, the increased consumption of craft beers and the use of dry-hopping techniques to produce beer with intense hop flavour encouraged new issues in hops science. Hops authenticity, characterization of established varieties that have been brought back due to their special capabilities, new hop varieties and production of beers that present new flavours or optimized composition of bioactive hop compounds are hot topics for brewing industry.

Molecular DNA analyses are the reference methods for discrimination and authentication of hops varieties, because chemical markers are strongly influenced by environmental conditions during plant growth (region, soil, harvest time, agricultural practices, environmental factors), cones processing and storage. Several DNA-based methods were developed targeting the plant phylogenetic studies and hops molecular identification, to circumvent the environmental variation of chemical markers. However, most of them are very complex and highly expensive. A simplified 7 Single Nucleotide Polymorphic (SNP) markers set with high-resolution melting analysis (HRMA) was applied to accurately genotype Portuguese hops from germplasm collection, wild hops and commercial samples. As SNP markers provide high likelihood of differentiating among varieties, the SNP/ HRMA approach that was applied demonstrated to be an effective way to discriminate Portuguese native and commercial reference genotypes. Furthermore, revealing that there is genetic differentiation of Portuguese native hops.

Eco-real-time-friendly-low-cost techniques to distinguish hop varieties, targeting authenticity purposes are of major relevance. NIR and MIR spectroscopy were successfully used for the discrimination and identification of hop varieties, aided by chemometric analysis, which proved to be valuable tools for hop discrimination. The NIR and MIR data analysis was performed using principal component analysis (PCA) (to detect outliers and find common patterns) and partial least squares discriminant analysis (PLS-DA) for development of discrimination models. A total of 165 samples from 33 commercial hop varieties (five for each hop variety) were analysed by both techniques. The available data were divided in calibration (70%) and validation (30%) sets in a random way and the NIR and MIR spectra were divided in five spectral regions. After the optimization of the best spectral regions and pre-processing methods, the test set was projected in the optimized PLS-DA calibration model to assess the percentage of correct predictions. One PLS-DA model was developed for each spectroscopic technique (NIR and MIR). A total of 94.2% and 96.6% correct hop varieties discrimination were obtained for NIR and MIR spectroscopy,

respectively, when a total of 165 commercial hop samples were analysed. The dendrogram obtained using NIR data grouped the hop varieties in two distinct clusters, which can be associated with the α-acids amount, one cluster for high amount of α-acids (>6%) and another cluster for the varieties that present lower α-acids content. Therefore, discrimination of 75 Portuguese native hops was done by NIR, since it was considered better than MIR to group hops by α-acids content. The majority of Portuguese hops presented low α-acids content and clustered together, some commercial varieties that present low content of α-acids also clustered with those Portuguese hops, while the varieties that present higher contents of α-acids clustered with 5 Portuguese hops. The correct hop varieties discrimination obtained for NIR was 89.9%. This represents the first work exclusively based on NIR and MIR spectroscopy techniques to hop cultivars fast discrimination within a highly diverse group of varieties. Compared with the molecular reference procedures, normally used for hops variety discrimination, these techniques are quicker, cost-effective, non-destructive and eco-friendly.

Discrimination between both hop populations (Portuguese native and commercial) was also performed by characterization of volatile profile extracted by headspace solid phase microextraction and analysed by gas chromatography coupled with mass spectrometry (HS-SPME-GC-MS). The volatile profile of Portuguese native hops can be useful to search for new varieties desired at modern beer trends. The chemometric technique of agglomerative hierarchical clustering (AHC) analysis pointed the natural groupings between samples. Moreover, chemometric techniques were relevant tools to understand the relationship between volatile profile and aroma attributes. In general, Portuguese native hops had different volatile profiles and aroma properties, in comparison with commercial hops selected from the most representative market varieties. Portuguese hops were related to resinous, spicy, and herbal aroma characteristics, while commercial hops were, in general, more citrus, fruity/ sweet, and floral. Most Portuguese native hops can be new alternatives for the development of a new varieties because they present completely new patterns, while other samples present similarities with commercial established varieties. PLS regression models provided information on the relationship between aroma characteristics and volatile profile, regardless of hop variety. PSL-DA model identified 12 volatile compounds responsible for the separation of commercial and Portuguese hops. Results pointed 2-methylpropanoic acid as the main compound to distinguish the tested hops, followed by α-amorphene, 2-methylbutyl 2-methylpropanoate, linalool, ethyl 4-methylpentanoate, 2-undecanone, 2-decanone, methyl geranate, perillen, 3-methylbutyl 2-methylpropanoate, β-ocimene and 3-methylbutanoic acid.

The brewing quality of some Portuguese native hops was evaluated. Four Portuguese native hops were selected based on their chemical composition (α-acids, β-acids,

xanthohumol and total oil quantification), sensory characteristics, genetic relatedness, and taking into consideration the technical limitations concerning the maximum number of hops to be used in the beers production. Dry-hopping techniques were selected because they have been widely used by the brewery industry to impart hop sensory impression to beers. Check-all-that-apply (CATA) sensory analyses demonstrated that those four selected Portuguese hops differed significantly in fruity (including citrus, green fruit, and sweet fruit), floral and woody attributes. GC-MS coupled with olfactometry detected 38 odour-active compounds, including terpenes and esters commonly described in commercial hops and sulphur compounds. After dry-hopping, some of these volatile compounds were extracted to beer over threshold detection. Portuguese hops promoted fruity and spicy notes, and no unpleasant sulphurous sensation was observed on dry-hopped beers.

Moreover, the relationships between aroma characteristics and volatile compounds were explored using chemometric PLS regressions: (i) in hops, after sensory and volatile profile analysis of Portuguese and commercial samples, successful models were found for relationship of the 12 volatile compounds responsible for the discrimination of the samples and sensory citrus, fruity/ sweet, and floral perceptions; (ii) from hops to beer, the impact of commercial variety Mandarina Bavaria (MBA) on the fruity-citrus intensity and volatile profile of dry-hopped beers was assessed. Successful models were obtained to predict total hoppy, citrus, green fruit, and sweet fruit characteristics of dry-hopped beers by equation regressions that consider only the content of four volatile compounds, myrcene, 2MB2MP, linalool, and α-humulene. It was also possible to observe that the initial volatile composition of beers influenced the extraction of volatile compounds from hops to beers. Concerning sensory profile, although beers presented statistical differences at the beginning, it grew fainter after 3 days of dry-hopping, up to 10 days, whereas at day 15, beers presented again significant differences of citrus descriptor.

Beside volatile compounds, dry-hopping techniques co-extract other hop components, including bioactive compounds, such as XN and α-acids, therefore, optimization of the extraction time and hop quantity to reach the highest yield of extraction of those compounds in beers was a key issue. For this purpose, beers were dry-hopped with different concentrations and contact time of commercial varieties Chinook (CHI), East Kent Goldings (EKG), and Tettnanger (TET). Reverse-phase liquid chromatography with ultra-violet diode array detection (RP-HPLC-UV-DAD) was performed to evaluate xanthohumol and α-acids contents, and chemometric nonlinear regression modeling were applied for data interpretation. Mathematic models that explain xanthohumol and α-acids extraction to beer were established, to calculate the minimum amount of hops to be added in the dry-hopping

process in order to reach a defined concentration of α-acids or XN in the final beer. The maximum efficiency of α-acids and xanthohumol extractions were reached at 13.5 days with dose rates of 147 and 13.9 mg/ L, respectively.

The antiproliferative effect of hop compounds (XN, IXN, ABA and IAA) was tested against human colorectal adenocarcinoma Caco-2 cells, using the pure compounds. The ranges of concentration tested, 0.313–20 μg/ mL for XN and IXN, 1.25–80 for ABA and 5.85–100 μg/ mL for IAA, took into consideration the average amounts occurring in different types of beers, and the possibility of applying different hopping practices that can increase their concentration. Xanthohumol did not show significant inhibitory effects in Caco-2 cells proliferation, whereas IXN showed significant inhibitory effect (ca. 25%) in Caco-2 cells proliferation at the concentrations of 10 and 20 μg/ mL. Moreover, dose-dependent responses were obtained for both ABA and IAA and IC50 values were calculated as 16.16 ± 3.11 μg/ mL (ABA) and 50,61 ± 13.08 μg/ mL (IAA). No Hopped Beer (NHB) matrix (containing all beers components except the ones originated from hops), lyophilized and reconstituted in the same volume of culture medium, presented 60% antiproliferative activity, being similar to that achieved by 40 μg/ mL ABA and by 100 μg/ mL IAA of pure compounds. A synergic effect was observed when pure compounds were added to the beer matrix. This effect was pronounced for ABA, which increased the activity of the beer matrix by 36% at 80 μg/ mL and for IAA with an increase of 28% in the activity of the matrix at 100 μg/ mL.

This thesis joins research on hops and beers. On one hand, a sequential methodologic action to support the enhancement of analytical methods for characterization and discrimination of wild hops was demonstrated. Studies performed with Portuguese native hops, highlighted the potential characteristic of new genotypes to be explored by the brewing industry, being used directly in beer production or as a genetic source for the development of new varieties. For this reason, the results obtained in this thesis generated attention of one of the biggest world companies of hops manufacture. A contract of material transfer agreement was signed concerning 7 samples of wild hops collected in nature, and the first analyses are already being done, including targeted genotyping by sequencing service (SeqSNP), screen hundreds up to 10,000 markers. The present study also contributes with evidence to an increment in the beer quality, due to better uncover the behaviour of extraction of key compounds in beer production, not only in organoleptic point of view, but also aiming the beverage beneficial health properties.

PART VI

Supplementary material

Appendix I: Sampling and samples treatment

A total of 178 hop samples were analyzed (Supplementary Table 1): 58 commercial varieties and 120 Portuguese native varieties, including 97 samples provided by Banco Português de Germoplasma Vegetal (coded as PTG) and 23 samples collected in nature (coded as PTW).

Leaves, cones and pellets (type 90) were used for different analysis (Supplementary Table 1). Leaves and cones were collected in natural environment, whereas pellets were acquired from local suppliers of raw materials for brewers. Leaves were dried in the field in desiccating silica-containing tubes and stored at - 20 ºC. Cones were dried at 60 °C. Dried cones and pellets were closed in vacuum bags and stored in the absence of light at 4 to 8 °C until the moment of analysis.

Prior to analysis, samples were thawed to room temperature and grounded at 10,000 RPM for 10 seconds to obtain a homogeneous powder in a Retsch GM 200 mill (Retsch GmbH, Haan, Germany).

Appendix II: Supplementary Tables

Supplementary Table 1. Hops leaves (L), cones (C) and pellets (P), used in each analysis and chapters (Ch).

Hops	Origin	Ch 2	Ch 3	Ch 4	Ch 5, 6 and 7
PTG01 to PTG21	PT	L *g*	C *ir, r*	C *v, s*	
PTG22	PT	L *g*	C *ir, r*	C *v, s*	C *to, v, s, o, bp*
PTG23 to PTG32	PT	L *g*	C *ir, r*	C *v, s*	C *to*
PTG33	PT	L *g*	C *ir, r*	C *v, s*	
PTG34 to PTG39	PT	L *g*	C *ir, r*	C *v, s*	C *to*
PTG40 and PTG41	PT	L *g*	C *ir, r*	C *v, s*	
PTG42 to PTG45	PT	L *g*	C *ir, r*	C *v, s*	C *to*
PTG46	PT	L *g*	C *ir, r*	C *v, s*	
PTG47 to PTG51	PT	L *g*	C *ir, r*	C *v, s*	C *to*
PTG52	PT	L *g*	C *ir, r*	C *v, s*	
PTG53 to PTG65	PT	L *g*	C *ir, r*	C *v, s*	C *to*
PTG66	PT	L *g*	C *ir, r*	C *v, s*	
PTG67 and PTG68	PT	L *g*			
PTG69	PT	L *g*			
PTG70 to PTG75	PT	L *g*			
PTG76	PT	L *g*	C *ir, r*		
PTG77 to PTG85	PT	L *g*			
PTG86	PT	L *g*			
PTG87 to PTG97	PT	L *g*			
PTW01	PT	L *g*	C *ir, r*	C *v, s*	
PTW02	PT	L *g*	C *ir, r*	C *v, s*	C *to, v, s, o, bp*
PTW03 and PTW04	PT	L *g*	C *ir, r*	C *v, s*	C *to*
PTW05 and PTW06	PT	L *g*	C *ir, r*	C *v, s*	
PTW07 and PTW08	PT	L *g*	C *ir, r*	C *v, s*	C *to, v, s, o, bp*
PTW09	PT	L *g*		C *v, s*	
PTW10	PT	L *g*			
PTW11	PT	L *g*			
PTW12 to PTW15	PT	L *g*			
PTW16	PT	L *g*	C *ir, r*		
PTW17	PT	L *g*			
PTW18	PT	L *g*			
PTW19 to PTW23	PT	L *g*			

Legend: genetics (*g*), infra-red (*ir*, NIR/ MIR), resins (*r*, α-acids, β-acids and xanthohumol), total oil (*to*), volatile compounds (*v*), sensory (*s*), sensory training (*st*), olfactometry (*o*), and beer production (*bp*).

Supplementary Table 1. Hops leaves (L), cones (C) and pellets (P), used in each analysis and chapters (Ch) (continued).

Hops	Origin	Ch 2	Ch 3	Ch 4	Ch 5, 6 and 7
ANA (Ariana)	DE				P *st*
BGO (Brewers Gold)	UK	L *g*	P *g, ir*	P *v, s*	
BRO (Bravo™)	US				P *st*
BRX (Bramling Cross)	UK		P *g, ir*	P *v, s*	
CAS (Cascade)	US	L *g*	P *g, ir*	P *v, s*	
CHA (Wye Challenger)	US	L *g*	P *g, ir*	P *v, s*	
CHI (Chinook)	US	L *g*	P *g, ir*	P *v, s*	P *r, bp*
CIT (Citra®)	US		P *g, ir*	P *v, s*	
CLU (College Cluster)	US	L *g*	P *g, ir*	P *v, s*	
COM (Comet)	US	L *g*			
CRY (Crystal)	US		P *g, ir*	P *v, s*	
EKG (East Kent Golding)	UK		P *g, ir*	P *v, s*	P *r, bp*
EKU (Ekuanot®)	US		P *g, ir*	P *v, s*	
ELL (Ella)	AU		P *g, ir*	P *v, s*	
FSP (Strisselspalter)	FR	L *g*			
FUG (Fuggle)	UK	L *g*	P *g, ir*	P *v, s*	
GOL (Goldings)	UK	L *g*	P *g, ir*	P *v, s*	
HAL (Hallertauer Mittelfrüh)	DE	L *g*	P *g, ir*	P *v, s*	
HBC (Hallertauer Blanc)	DE				P *st*
HEB (Hersbrucker Spät)	DE	L *g*	P *g, ir*	P *v, s*	P *st*
HKS (Herkules)	DE				P *st*
HMG (Hallertauer Magnum)	DE	L *g*	P *g, ir*	P *v, s*	P *st*
HMN (Hüll Melon)	DE				P *st*
HTR (Hallertauer Tradition)	DE	L *g*	P *g, ir*	P *v, s*	P *st*
HTU (Hallertauer Taurus)	DE	L *g*			P *st*
LUB (Lubelski)	PL	L *g*			
MAR (Marynka)	PL	L *g*			
MBA (Mandarina Bavaria)	DE				P *st v, s, bp*
MOS (Mosaic®)	US		P *g, ir*	P *v, s*	
MTH (Mount Hood)	US	L *g*	P *g, ir*	P *v, s*	
NBR (Northern Brewer)	UK	L *g*	P *g, ir*	P *v, s*	
NSN (Nelson Sauvin™)	NZ		P *g, ir*	P *v, s*	
NUG (Nugget)	US	L *g*	P *g, ir*	P *v, s*	P *st*
OPL (Opal)	DE				P *st*
PER (Perle)	DE	L *g*	P *g, ir*	P *v, s*	
PLA (Polaris)	DE			P *v, s*	P *st*
POR (Pride of Ringwood)	AU	L *g*			

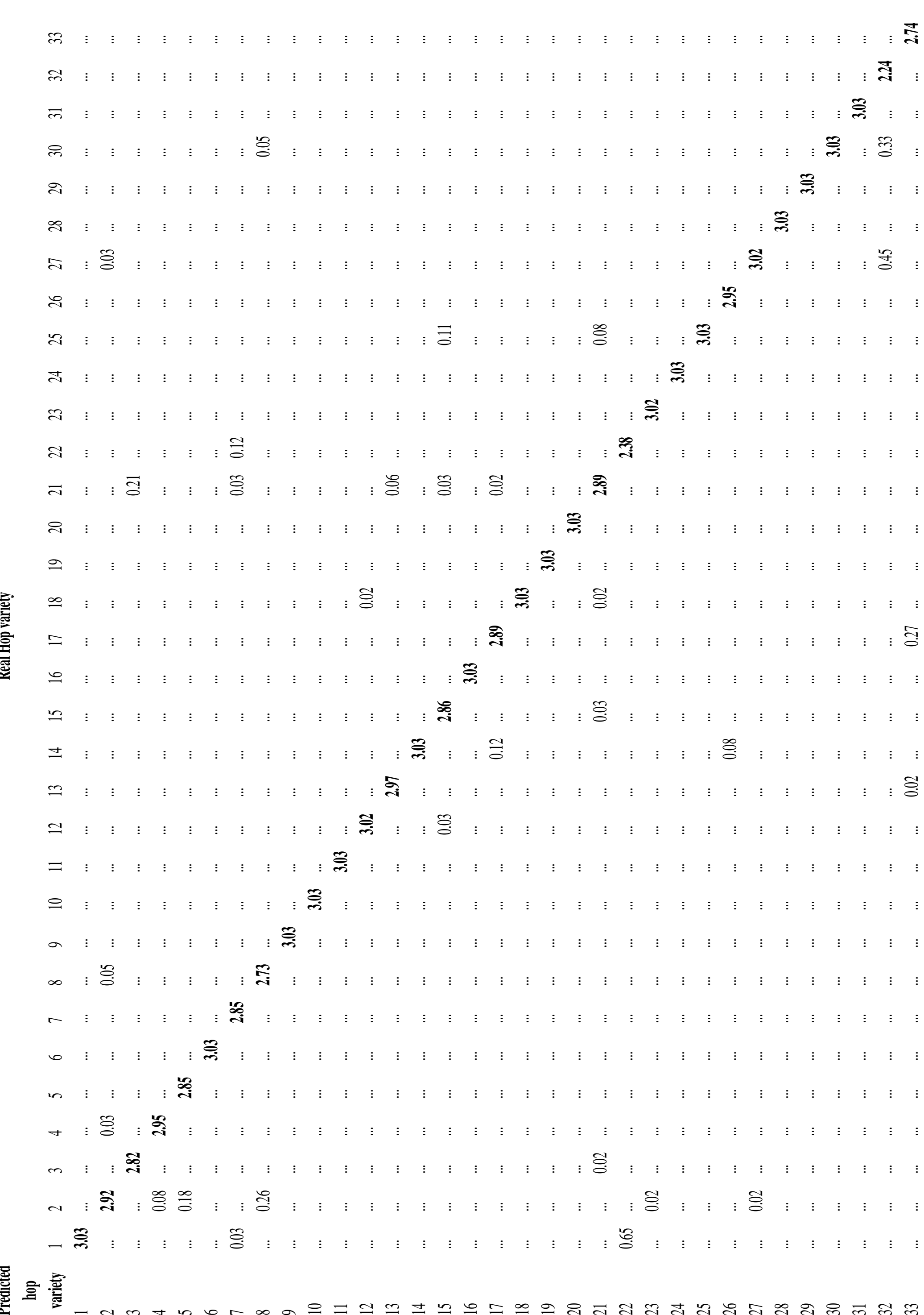
Legend: genetics (*g*), infra-red (*ir*, NIR/ MIR), resins (*r*, α-acids, β-acids and xanthohumol), total oil (*to*), volatile compounds (*v*), sensory (*s*), sensory training (*st*), olfactometry (*o*), and beer production (*bp*).

Supplementary Table 1. Hops leaves (L), cones (C) and pellets (P), used in each analysis and chapters (Ch) (continued).

Hops	Origin	Ch 2	Ch 3	Ch 4	Ch 5, 6 and 7
SAZ (Saaz Osvaldov klon 72)	CZ	L *g*	P *g, ir*	P *v, s*	
SGB (Bobek)	SI	L *g*	P *g, ir*	P *v, s*	
SGC (Celeia)	SI	L *g*			
SGD (Smaragd)	DE				P *st*
SIM (Simcoe®)	US		P *g, ir*	P *v, s*	
SIR (Saphir)	DE	L *g*			
SLD (Sladek)	CZ	L *g*			
SPA (Spalter)	DE	L *g*			
SSA (Aurora)	SI	L *g*			
SSE (Spalter Select)	DE	L *g*	P *g, ir*	P *v, s*	
SSG (Styrian Savinjski Golding)	SI	L *g*			
SUM (Summit®)	US		P *g, ir*	P *v, s*	P *bp*
TET (Tettnanger)	DE	L *g*	P *g, ir*	P *v, s*	P *r, bp*
TOM (Tomahawk®)	US		P *g, ir*	P *v, s*	
TRG (Wye Target)	US	L *g*	P *g, ir*	P *v, s*	
VG1 (Amarillo®)	US		P *g, ir*	P *v, s*	
WIL (Willamette)	US	L *g*	P *g, ir*	P *v, s*	

Legend: genetics (*g*), infra-red (*ir*, NIR/ MIR), resins (*r*, α-acids, β-acids and xanthohumol), total oil (*to*), volatile compounds (*v*), sensory (*s*), sensory training (*st*), olfactometry (*o*), and beer production (*bp*).

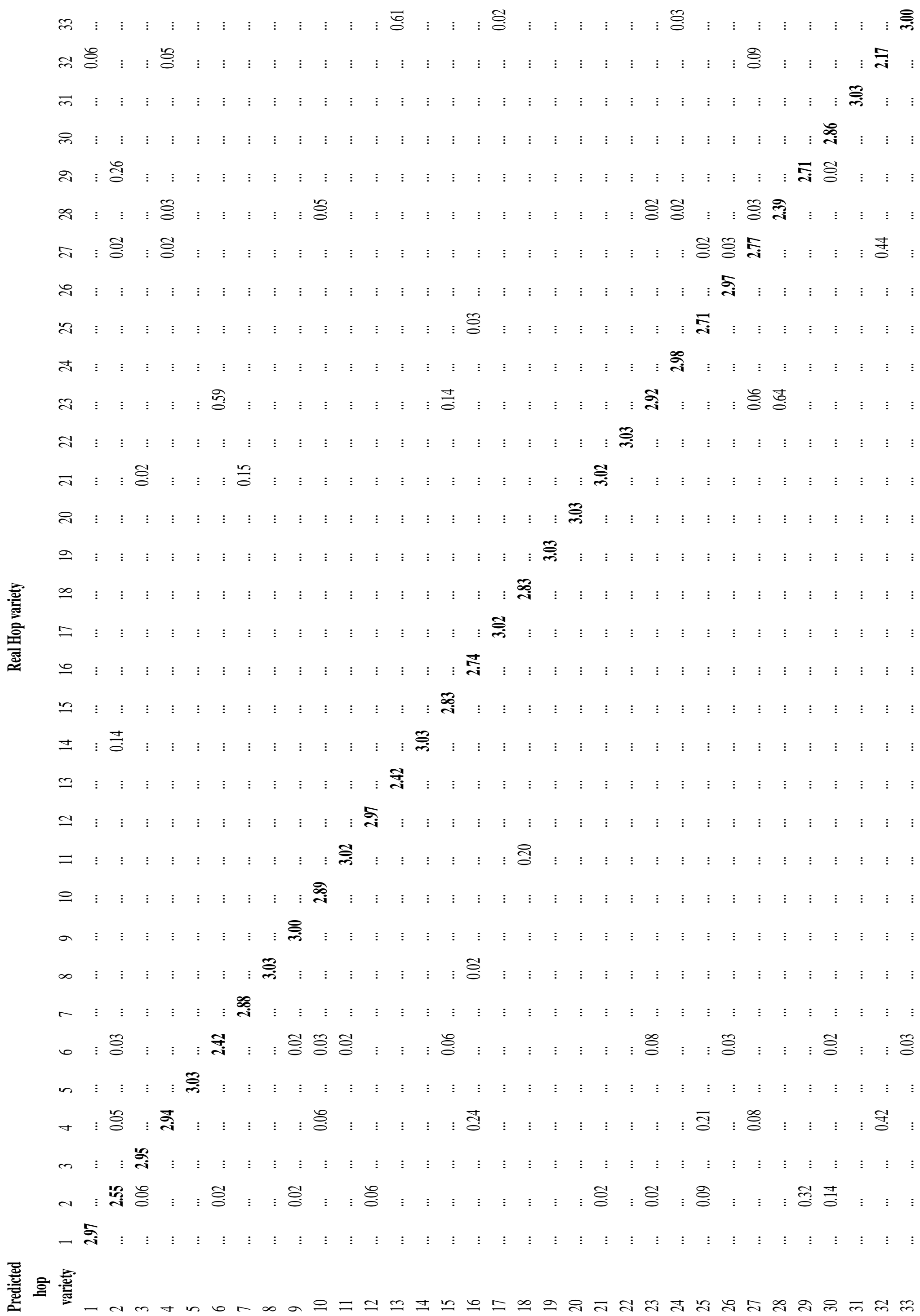
Supplementary Table 2. Confusion matrix obtained with PLS-DA of NIR spectra.



Predicted hop variety	Real Hop variety																																
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33
1	**3.03**	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
2	...	**2.92**	...	0.03	...	...	...	0.05	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	0.03	...	...	...	...	...	...
3	...	...	**2.82**	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	0.21	...	...	...	...	...	...	...	...	...	...	...	...
4	...	0.08	...	**2.95**	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
5	...	0.18	...	...	**2.85**	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
6	...	...	...	...	...	**3.03**	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
7	0.03	...	...	...	...	...	**2.85**	...	...	...	...	...	...	...	...	...	...	...	...	...	0.03	0.12	...	...	...	...	...	...	...	...	...	...	...
8	...	0.26	...	...	...	...	...	**2.73**	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	0.05	...	...	...
9	...	...	...	...	...	...	...	...	**3.03**	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
10	...	...	...	...	...	...	...	...	...	**3.03**	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
11	...	...	...	...	...	...	...	...	...	...	**3.03**	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
12	...	...	...	...	...	...	...	...	...	...	...	**3.02**	...	...	...	...	...	0.02	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
13	...	...	...	...	...	...	...	...	...	...	...	...	**2.97**	...	...	...	...	...	...	...	0.06	...	...	...	...	...	...	...	...	...	...	...	...
14	...	...	...	...	...	...	...	...	...	...	...	...	...	**3.03**	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
15	...	...	...	...	...	...	...	...	...	...	...	0.03	...	...	**2.86**	...	...	...	...	...	0.03	...	...	...	0.11	...	...	...	...	...	...	...	...
16	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	**3.03**	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
17	...	...	...	...	...	...	...	...	...	...	...	...	...	0.12	...	...	**2.89**	...	...	...	0.02	...	...	...	...	...	...	...	...	...	...	...	...
18	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	**3.03**	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
19	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	**3.03**	...	...	...	...	...	...	...	...	...	...	...	...	...	...
20	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	**3.03**	...	...	...	...	...	...	...	...	...	...	...	...	...
21	...	...	0.02	...	...	...	...	...	...	...	...	...	...	...	0.03	...	...	0.02	...	...	**2.89**	...	...	...	0.08	...	...	...	...	...	...	...	...
22	0.65	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	**2.38**	...	...	...	...	...	...	...	...	...	...	...
23	...	0.02	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	**3.02**	...	...	...	...	...	...	...	...	...	...
24	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	**3.03**	...	...	...	...	...	...	...	...	...
25	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	**3.03**	...	...	...	...	...	...	...	...
26	...	...	...	...	...	...	...	...	...	...	...	...	...	0.08	...	...	...	...	...	...	...	...	...	...	...	**2.95**	...	...	...	...	...	...	...
27	...	0.02	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	**3.02**	...	...	...	...	...	...
28	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	**3.03**	...	...	...	...	...
29	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	**3.03**	...	...	...	...
30	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	**3.03**	...	...	...
31	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	**3.03**	...	...
32	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	0.45	...	...	0.33	...	**2.24**	...
33	...	...	...	...	...	...	...	...	...	...	...	...	0.02	...	...	...	0.27	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	**2.74**

Legend: 1- FUG; 2-HTR; 3- CAS; 4-SGB; 5- HAL; 6- CHI; 7- EKG; 8- TET; 9- ELL, 10- NUG; 11- EKU; 12- SUM; 13- NSN; 14- MTH; 15- CIT; 16- CLU; 17- CRY; 18- TOM; 19- BRX; 20- BGO; 21- CHA; 22- GOL; 23- MOS; 24- HMG; 25- WIL; 26- TRG; 27- NBR; 28- SIM; 29- SSE; 30- SAZ; 31- HEB; 32- PER; 33- VG1; ...- 0.00.

Supplementary Table 3. Confusion matrix obtained with PLS-DA of MIR spectra.



Predicted hop variety	Real Hop variety																																
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33
1	**2.97**	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	0.06	...
2	...	**2.55**	...	0.05	...	0.03	...	...	...	...	...	...	...	0.14	...	...	...	...	...	...	...	...	...	...	...	...	0.02	...	0.26	...	...	...	...
3	...	0.06	**2.95**	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	0.02	...	...	...	...	...	...	...	...	...	...	...	...
4	...	...	...	**2.94**	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	0.02	0.03	...	...	...	0.05	...
5	...	...	...	...	**3.03**	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
6	...	0.02	...	...	...	**2.42**	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	0.59	...	...	...	...	...	...	...	...	...	...
7	...	...	...	...	...	...	**2.88**	...	...	...	...	...	...	...	...	...	...	...	...	...	0.15	...	...	...	...	...	...	...	...	...	...	...	...
8	...	...	...	...	...	...	...	**3.03**	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
9	...	0.02	...	...	...	0.02	...	...	**3.00**	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
10	...	...	...	0.06	...	0.03	...	...	...	**2.89**	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	0.05	...	...	...	...	...
11	...	...	...	...	...	0.02	...	...	...	...	**3.02**	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
12	...	0.06	...	...	...	...	...	...	...	...	...	**2.97**	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
13	...	...	...	...	...	...	...	...	...	...	...	...	**2.42**	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	0.61
14	...	...	...	...	...	...	...	...	...	...	...	...	...	**3.03**	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
15	...	...	...	...	...	0.06	...	...	...	...	...	...	...	...	**2.83**	...	...	...	...	...	...	...	0.14	...	...	...	...	...	...	...	...	...	...
16	...	...	...	0.24	...	...	...	0.02	...	...	...	...	...	...	...	**2.74**	...	...	...	...	...	...	...	...	0.03	...	...	...	...	...	...	...	...
17	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	**3.02**	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	0.02
18	...	...	...	...	...	...	...	...	...	...	0.20	...	...	...	...	...	...	**2.83**	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
19	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	**3.03**	...	...	...	...	...	...	...	...	...	...	...	...	...	...
20	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	**3.03**	...	...	...	...	...	...	...	...	...	...	...	...	...
21	...	0.02	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	**3.02**	...	...	...	...	...	...	...	...	...	...	...	...
22	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	**3.03**	...	...	...	...	...	...	...	...	...	...	...
23	...	0.02	...	...	...	0.08	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	**2.92**	...	...	...	...	0.02	...	...	...	...	...
24	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	**2.98**	...	...	...	0.02	...	...	...	...	0.03
25	...	0.09	...	0.21	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	**2.71**	...	0.02	...	...	...	...	...	...
26	...	...	...	...	...	0.03	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	**2.97**	0.03	...	...	...	...	...	...
27	...	...	...	0.08	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	0.06	...	...	...	**2.77**	0.03	...	...	...	0.09	...
28	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	0.64	...	...	...	...	**2.39**	...	...	...	...	...
29	...	0.32	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	**2.71**	...	...	...	...
30	...	0.14	...	...	...	0.02	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	0.02	**2.86**	...	...	...
31	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	**3.03**	...	...
32	...	...	...	0.42	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	0.44	...	...	...	...	**2.17**	...
33	...	...	...	...	...	0.03	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	**3.00**

Legend: 1- FUG; 2-HTR; 3- CAS; 4-SGB; 5- HAL; 6- CHI; 7- EKG; 8- TET; 9- ELL, 10- NUG; 11- EKU; 12- SUM; 13- NSN; 14- MTH; 15- CIT; 16- CLU; 17- CRY; 18- TOM; 19- BRX; 20- BGO; 21- CHA; 22- GOL; 23- MOS; 24- HMG; 25- WIL; 26- TRG; 27- NBR; 28- SIM; 29- SSE; 30- SAZ; 31- HEB; 32- PER; 33- VG1; ...- 0.00.

Supplementary Table 4. A contingency table of the proportions of selection by 16 semi-trained panellists across all 109 hop samples for individual terms of the Check-All-That-Apply (CATA) question.

Hop samples	Citrus	Fruity/ Sweet	Floral	Spicy	Resinous	Herbal
Commercial						
VG1	0.121 abcde	0.212 ab	0.061 a	0.121 ab	0.121 abc	0.091 abc
BGO	0.091 abcd	0.303 ab	0.121 a	0.152 ab	0.212 abc	0.182 abc
SGB	0.000 a	0.182 ab	0.091 a	0.030 ab	0.121 abc	0.061 ab
BRX	0.091 abcd	0.273 ab	0.091 a	0.273 ab	0.364 abc	0.182 abc
CAS	0.455 e	0.121 ab	0.061 a	0.121 ab	0.030 ab	0.000 a
CHA	0.152 abcde	0.394 ab	0.121 a	0.242 ab	0.152 abc	0.152 abc
CHI	0.091 abcd	0.333 ab	0.182 a	0.061 ab	0.091 abc	0.182 abc
CIT	0.424 de	0.152 ab	0.091 a	0.030 ab	0.000 a	0.061 ab
CLU	0.030 ab	0.303 ab	0.091 a	0.121 ab	0.182 abc	0.091 abc
CRY	0.030 ab	0.091 ab	0.182 a	0.182 ab	0.061 ab	0.242 abc
EKG	0.030 ab	0.333 ab	0.061 a	0.091 ab	0.212 abc	0.303 abc
ELL	0.061 abc	0.242 ab	0.091 a	0.000 a	0.030 ab	0.121 abc
EKU	0.303 abcde	0.152 ab	0.152 a	0.121 ab	0.030 ab	0.061 ab
FUG	0.030 ab	0.152 ab	0.030 a	0.091 ab	0.333 abc	0.364 abc
GOL	0.182 abcde	0.242 ab	0.121 a	0.121 ab	0.333 abc	0.121 abc
HEB	0.030 ab	0.212 ab	0.121 a	0.212 ab	0.333 abc	0.182 abc
MTH	0.030 ab	0.333 ab	0.212 a	0.212 ab	0.152 abc	0.212 abc
HMG	0.030 ab	0.273 ab	0.121 a	0.212 ab	0.242 abc	0.152 abc
HAL	0.121 abcde	0.273 ab	0.121 a	0.212 ab	0.182 abc	0.303 abc
MOS	0.424 de	0.303 ab	0.212 a	0.091 ab	0.091 abc	0.091 abc
NBR	0.212 abcde	0.364 ab	0.121 a	0.242 ab	0.121 abc	0.061 ab
NSN	0.242 abcde	0.303 ab	0.152 a	0.091 ab	0.061 ab	0.091 abc
NUG	0.182 abcde	0.212 ab	0.030 a	0.091 ab	0.152 abc	0.091 abc
PER	0.333 abcde	0.303 ab	0.152 a	0.212 ab	0.182 abc	0.091 abc
PLA	0.061 abc	0.485 b	0.212 a	0.121 ab	0.121 abc	0.121 abc
SSE	0.061 abc	0.273 ab	0.061 a	0.091 ab	0.333 abc	0.212 abc
SAZ	0.182 abcde	0.182 ab	0.091 a	0.212 ab	0.273 abc	0.242 abc
SIM	0.333 abcde	0.273 ab	0.182 a	0.121 ab	0.091 abc	0.091 abc
SUM	0.212 abcde	0.182 ab	0.121 a	0.152 ab	0.121 abc	0.212 abc
TRG	0.030 ab	0.333 ab	0.061 a	0.242 ab	0.394 bc	0.212 abc
TET	0.152 abcde	0.364 ab	0.152 a	0.030 ab	0.152 abc	0.091 abc
TOM	0.303 abcde	0.273 ab	0.091 a	0.121 ab	0.152 abc	0.121 abc
HTR	0.303 abcde	0.242 ab	0.242 a	0.182 ab	0.000 a	0.212 abc
WIL	0.030 ab	0.242 ab	0.182 a	0.121 ab	0.303 abc	0.182 abc
Portuguese						
PTG1	0.273 abcde	0.394 ab	0.061 a	0.121 ab	0.182 abc	0.091 abc
PTG2	0.121 abcde	0.394 ab	0.061 a	0.212 ab	0.091 abc	0.030 ab

Cochran's Q test was performed to determine whether the proportions of selection by the semi-trained panel for individual terms of the CATA question differed as a function of hop sample. Only significant terms ($p < 0.050$) were shown. Post-hoc multiple pairwise comparisons were performed using Marascuilo's test. The proportions with different letters within each column represent a significant difference at $p < 0.050$.

Supplementary Table 4. A contingency table of the proportions of selection by 16 semi-trained panellists across all 109 hop samples for individual terms of the Check-All-That-Apply (CATA) question (continued).

Hop samples	Citrus	Fruity/ Sweet	Floral	Spicy	Resinous	Herbal
Portuguese						
PTG3	0.364 bcde	0.242 ab	0.091 a	0.212 ab	0.152 abc	0.152 abc
PTG4	0.182 abcde	0.182 ab	0.212 a	0.242 ab	0.212 abc	0.152 abc
PTG5	0.212 abcde	0.303 ab	0.121 a	0.152 ab	0.091 abc	0.121 abc
PTG6	0.030 ab	0.000 a	0.030 a	0.000 a	0.364 abc	0.394 bc
PTG7	0.182 abcde	0.242 ab	0.091 a	0.121 ab	0.061 ab	0.061 ab
PTG8	0.212 abcde	0.182 ab	0.121 a	0.273 ab	0.121 abc	0.273 abc
PTG9	0.242 abcde	0.273 ab	0.182 a	0.212 ab	0.061 ab	0.030 ab
PTG10	0.091 abcd	0.182 ab	0.121 a	0.212 ab	0.242 abc	0.303 abc
PTG11	0.273 abcde	0.182 ab	0.121 a	0.212 ab	0.182 abc	0.152 abc
PTG12	0.061 abc	0.212 ab	0.152 a	0.212 ab	0.242 abc	0.242 abc
PTG13	0.182 abcde	0.212 ab	0.091 a	0.242 ab	0.061 ab	0.091 abc
PTG14	0.030 ab	0.273 ab	0.061 a	0.182 ab	0.152 abc	0.212 abc
PTG15	0.000 a	0.152 ab	0.000 a	0.000 a	0.242 abc	0.333 abc
PTG16	0.000 a	0.121 ab	0.121 a	0.030 ab	0.242 abc	0.303 abc
PTG17	0.182 abcde	0.273 ab	0.152 a	0.121 ab	0.121 abc	0.121 abc
PTG18	0.303 abcde	0.152 ab	0.182 a	0.152 ab	0.152 abc	0.091 abc
PTG19	0.212 abcde	0.333 ab	0.121 a	0.182 ab	0.061 ab	0.000 a
PTG20	0.061 abc	0.061 a	0.030 a	0.242 ab	0.394 bc	0.303 abc
PTG21	0.152 abcde	0.212 ab	0.091 a	0.242 ab	0.121 abc	0.273 abc
PTG22	0.152 abcde	0.273 ab	0.061 a	0.333 ab	0.061 ab	0.091 abc
PTG23	0.061 abc	0.242 ab	0.121 a	0.030 ab	0.212 abc	0.242 abc
PTG24	0.212 abcde	0.303 ab	0.091 a	0.091 ab	0.030 ab	0.061 ab
PTG25	0.273 abcde	0.212 ab	0.152 a	0.182 ab	0.091 abc	0.212 abc
PTG26	0.212 abcde	0.303 ab	0.212 a	0.364 b	0.091 abc	0.121 abc
PTG27	0.212 abcde	0.242 ab	0.091 a	0.242 ab	0.273 abc	0.152 abc
PTG28	0.030 ab	0.242 ab	0.152 a	0.182 ab	0.303 abc	0.152 abc
PTG29	0.152 abcde	0.152 ab	0.273 a	0.152 ab	0.242 abc	0.182 abc
PTG30	0.030 ab	0.091 ab	0.030 a	0.121 ab	0.455 c	0.394 bc
PTG31	0.303 abcde	0.242 ab	0.061 a	0.182 ab	0.152 abc	0.061 ab
PTG32	0.061 abc	0.152 ab	0.121 a	0.212 ab	0.273 abc	0.152 abc
PTG33	0.091 abcd	0.242 ab	0.152 a	0.061 ab	0.182 abc	0.212 abc
PTG34	0.030 ab	0.212 ab	0.091 a	0.182 ab	0.212 abc	0.212 abc
PTG35	0.121 abcde	0.061 a	0.091 a	0.212 ab	0.212 abc	0.303 abc
PTG36	0.182 abcde	0.364 ab	0.091 a	0.091 ab	0.091 abc	0.091 abc
PTG37	0.121 abcde	0.182 ab	0.091 a	0.182 ab	0.091 abc	0.242 abc
PTG38	0.061 abc	0.152 ab	0.091 a	0.242 ab	0.242 abc	0.394 bc
PTG39	0.000 a	0.091 ab	0.091 a	0.121 ab	0.212 abc	0.455 c

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Supplementary Table 4. A contingency table of the proportions of selection by 16 semi-trained panellists across all 109 hop samples for individual terms of the Check-All-That-Apply (CATA) question (continued).

Hop samples	Citrus	Fruity/ Sweet	Floral	Spicy	Resinous	Herbal
Portuguese						
PTG40	0.030 ab	0.182 ab	0.061 a	0.212 ab	0.273 abc	0.333 abc
PTG41	0.000 a	0.000 a	0.030 a	0.030 ab	0.333 abc	0.455 c
PTG42	0.152 abcde	0.182 ab	0.091 a	0.152 ab	0.182 abc	0.182 abc
PTG43	0.121 abcde	0.212 ab	0.030 a	0.121 ab	0.182 abc	0.091 abc
PTG44	0.000 a	0.061 a	0.061 a	0.091 ab	0.364 abc	0.333 abc
PTG45	0.030 ab	0.152 ab	0.030 a	0.061 ab	0.333 abc	0.303 abc
PTG46	0.000 a	0.152 ab	0.152 a	0.061 ab	0.212 abc	0.273 abc
PTG47	0.061 abc	0.152 ab	0.061 a	0.091 ab	0.182 abc	0.182 abc
PTG48	0.000 a	0.121 ab	0.061 a	0.061 ab	0.182 abc	0.303 abc
PTG49	0.333 abcde	0.273 ab	0.121 a	0.212 ab	0.091 abc	0.121 abc
PTG50	0.121 abcde	0.273 ab	0.061 a	0.303 ab	0.152 abc	0.182 abc
PTG51	0.030 ab	0.061 a	0.061 a	0.000 a	0.333 abc	0.303 abc
PTG52	0.030 ab	0.273 ab	0.242 a	0.182 ab	0.242 abc	0.273 abc
PTG53	0.182 abcde	0.333 ab	0.182 a	0.152 ab	0.152 abc	0.121 abc
PTG54	0.061 abc	0.303 ab	0.182 a	0.121 ab	0.121 abc	0.182 abc
PTG55	0.212 abcde	0.182 ab	0.121 a	0.152 ab	0.182 abc	0.152 abc
PTG56	0.061 abc	0.091 ab	0.061 a	0.061 ab	0.121 abc	0.364 abc
PTG57	0.121 abcde	0.121 ab	0.091 a	0.242 ab	0.212 abc	0.273 abc
PTG58	0.061 abc	0.212 ab	0.061 a	0.091 ab	0.242 abc	0.333 abc
PTG59	0.152 abcde	0.242 ab	0.121 a	0.091 ab	0.121 abc	0.152 abc
PTG60	0.030 ab	0.121 ab	0.030 a	0.364 b	0.152 abc	0.182 abc
PTG61	0.152 abcde	0.273 ab	0.091 a	0.152 ab	0.212 abc	0.091 abc
PTG62	0.000 a	0.121 ab	0.000 a	0.121 ab	0.333 abc	0.303 abc
PTG63	0.061 abc	0.242 ab	0.121 a	0.242 ab	0.121 abc	0.242 abc
PTG64	0.030 ab	0.182 ab	0.030 a	0.121 ab	0.212 abc	0.212 abc
PTG65	0.061 abc	0.212 ab	0.152 a	0.061 ab	0.303 abc	0.273 abc
PTG66	0.000 a	0.061 a	0.061 a	0.091 ab	0.394 bc	0.394 bc
PTW1	0.121 abcde	0.333 ab	0.061 a	0.121 ab	0.091 abc	0.242 abc
PTW2	0.333 abcde	0.212 ab	0.182 a	0.273 ab	0.182 abc	0.061 ab
PTW3	0.121 abcde	0.212 ab	0.061 a	0.333 ab	0.182 abc	0.273 abc
PTW4	0.364 bcde	0.273 ab	0.091 a	0.152 ab	0.121 abc	0.152 abc
PTW5	0.364 bcde	0.091 ab	0.212 a	0.212 ab	0.152 abc	0.121 abc
PTW6	0.152 abcde	0.303 ab	0.212 a	0.061 ab	0.091 abc	0.152 abc
PTW7	0.394 cde	0.212 ab	0.182 a	0.152 ab	0.182 abc	0.030 ab
PTW8	0.030 ab	0.273 ab	0.030 a	0.333 ab	0.212 abc	0.152 abc
PTW9	0.000 a	0.000 a	0.121 a	0.121 ab	0.303 abc	0.394 c
p	<0.001	<0.001	0.016	<0.001	<0.001	<0.001

Cochran's Q test was performed to determine whether the proportions of selection by the semi-trained panel for individual terms of the CATA question differed as a function of hop sample. Only significant terms ($p < 0.050$) were shown. Post-hoc multiple pairwise comparisons were performed using Marascuilo's test. The proportions with different letters within each column represent a significant difference at $p < 0.050$.

PART VIII

REFERENCES

References

1. Schönberger C, Kostelecky T. 125th Anniversary Review: The Role of Hops in Brewing. J Inst Brew. 2011;117(3):259-67.
2. Almaguer C, Schoenberger C, Gastl M, Arendt EK, Becker T. Humulus lupulus - a story that begs to be told. A review. J I Brewing. 2014;120(4):289-314.
3. Barth-Haas G. The Barth Report. Nuremberg; 2018.
4. Patzak J, Nesvadba V, Henychová A, Krofta K. Assessment of the genetic diversity of wild hops (Humulus lupulus L.) in Europe using chemical and molecular analyses. Biochemical Systematics and Ecology. 2010;38(2):136-45.
5. Krofta K, Nesvadba V, Patzak J. Utilization of wild hops testation for extention of genetic sources for breeding. Rostlinna Vyroba. 1998;44(7):313-20.
6. Healey J. The Hops List: 265 Beer Hop Varieties From Around the World. Melbourne2016. 550 p.
7. Barth-Haas G. Barth-Haas Hops Companion: A Guide to the Varieties of Hops and Hop Products. 3th ed. Yakima: John I. Haas, Inc.; 2016. 190 p.
8. Steenackers B, De Cooman L, De Vos D. Chemical transformations of characteristic hop secondary metabolites in relation to beer properties and the brewing process: A review. Food Chemistry. 2015;172:742-56.
9. Korbecka-Glinka G, Skomra U, Olszak-Przybys H. Cultivar identification in dry hop cones and pellets using microsatellite loci. Eur Food Res Technol. 2016;242(9):1599-605.
10. Henning JA, Coggins J, Peterson M. Simple SNP-based minimal marker genotyping for Humulus lupulus L. identification and variety validation. BMC research notes. 2015;8:542-.
11. Rocha F. Distribuição e ecologia do lúpulo (Humulus lupulus L.) em Portugal: Universidade do Minho; 2005.
12. Rettberg N, Biendl M, Garbe L-A. Hop Aroma and Hoppy Beer Flavor: Chemical Backgrounds and Analytical Tools—A Review. J Am Soc Brew Chem. 2018;76(1):1-20.
13. Donaldson BR, Bamforth CW, Heymann H. Sensory Descriptive Analysis and Free-Choice Profiling of Thirteen Hop Varieties as Whole Cones and After Dry Hopping of Beer. J Am Soc Brew Chem. 2012;70(3):176-81.
14. Mikyška A, Olšovská J, Slabý M, Štěrba K, Čerenak A, Košir IJ, et al. Analytical and sensory profiles of Slovenian and Czech hop genotypes in single hopped beers. J I Brewing. 2018;124(3):209-21.
15. Mitter W, Cocuzza S. Dry hopping - A study of various parameters - Consequences of the applied dosing method. Brewing and Beverage Industry International. 2013;4:70 - 4.
16. Vollmer DM, Lafontaine SR, Shellhammer TH. Aroma Extract Dilution Analysis of Beers Dry-Hopped with Cascade, Chinook, and Centennial. J Am Soc Brew Chem. 2018;76(3):190-8.
17. Haslbeck K, Minkenberg D, Coelhan M. Investigations into the Transfer Rate of Volatile Compounds in Dry Hopping Using an Octanol-Water Partition Coefficient Model. J Am Soc Brew Chem. 2018;76(3):169-77.
18. Sharp DC, Qian Y, Shellhammer G, Shellhammer TH. Contributions of Select Hopping Regimes to the Terpenoid Content and Hop Aroma Profile of Ale and Lager Beers. J Am Soc Brew Chem. 2017;75(2):93-100.
19. Lafontaine SR, Shellhammer TH. Impact of static dry-hopping rate on the sensory and analytical profiles of beer. J I Brewing. 2018;124(4):434-42.
20. De Keukeleire D, Milligan SR, Kalita JC, Pocock V, De Cooman L, Heyerick A, et al., editors. Prenylated hop flavonoids are key agents in relation to health-beneficial properties of beer.

Proceedings of the 28th International Congress of the European Brewery Convention; 2001 May, 12 -17; Budapest.
21. Viegas O, Zegura B, Pezdric M, Novak M, Ferreira IMPLVO, Pinho O, et al. Protective effects of xanthohumol against the genotoxicity of heterocyclic aromatic amines MeIQx and PhIP in bacteria and in human hepatoma (HepG2) cells. Food Chem Toxicol. 2012;50(3-4):949-55.
22. Polak J, Bartoszek M, Stanimirova I. A study of the antioxidant properties of beers using electron paramagnetic resonance. Food Chemistry. 2013;141(3):3042-9.
23. Stevens JF, Ivancic M, Hsu VL, Deinzer ML. Prenylflavonoids from Humulus lupulus. Phytochemistry. 1997;44(8):1575-85.
24. Stevens JF, Taylor AW, Clawson JE, Deinzer ML. Fate of xanthohumol and related prenylflavonoids from hops to beer. Journal of Agricultural and Food Chemistry. 1999;47:2421-8.
25. Hieronymus S. For the love of hops : the practical guide to aroma, bitterness, and the culture of hops: Brewers Publications; 2012. 321- p.
26. Eyck LAT, Gehring D. The hop grower's handbook : the essential guide for sustainable, small-scale production for home and market. New York: Chelsea Green Publishing; 2015. 279- p.
27. Horreo JL, Peredo EL, Olmedo JL, Valladares JE, García E, Revilla MA. Genetic diversity inferred from microsatellites of wild hops in Galicia (Spain). BrewingScience. 2014;67(11-12):132-6.
28. Murakami a, Darby P, Javornik B, Pais MSS, Seigner E, Lutz a, et al. Molecular phylogeny of wild hops, Humulus lupulus L. Heredity. 2006;97(1):66-74.
29. Hough JS, Briggs DE, Stevens R, Young TW. The Chemistry of Hop Constituents. Boston, MA: Springer US; 1982. p. 422-55.
30. Prencipe FP, Brighenti V, Rodolfi M, Mongelli A, dall'Asta C, Ganino T, et al. Development of a new high-performance liquid chromatography method with diode array and electrospray ionization-mass spectrometry detection for the metabolite fingerprinting of bioactive compounds in Humulus lupulus L. Journal of Chromatography A. 2014;1349:50-9.
31. Kankolongo Cibaka M-L, Gros J, Nizet S, Collin S. Quantitation of Selected Terpenoids and Mercaptans in the Dual-Purpose Hop Varieties Amarillo, Citra, Hallertau Blanc, Mosaic, and Sorachi Ace. J Agric Food Chem. 2015;63(11):3022-30.
32. Salanţă L, Tofană M, Socaci S, Mudura E, Pop C, Pop A, et al. Evaluation and comparison of aroma volatile compounds in hop varieties grown in Romania. Romanian Biotechnological Letters. 2015;20(6):11049-56.
33. Van Opstaele F, De Rouck G, De Clippeleer J, Aerts G, De Cooman L. Analytical and sensory assessment of hoppy aroma and bitterness of conventionally hopped and advanced hopped Pilsner beers. J Inst Brew 2011;110(4):445-58.
34. Eri S, Khoo BK, Lech J, Hartman TG. Direct thermal desorption−gas chromatography and gas chromatography−mass spectrometry profiling of hop (Humulus lupulus L.) essential oils in support of varietal characterization. Journal of Agricultural and Food Chemistry. 2000;48(4):1140-9.
35. Taniguchi Y, Taniguchi H, Yamada M, Matsukura Y, Koizumi H, Furihata K, et al. Analysis of the components of hard resin in hops (Humulus lupulus L.) and structural elucidation of their transformation products formed during the brewing process. Journal of Agricultural and Food Chemistry. 2014;62(47):11602-12.
36. Palmer JJ. How to Brew: Everything You Need To Know To Brew Beer Right The First Time: Brewers Publications; 2006. 500 p.
37. Roberts TR, Wilson RJH. Hops. In: Priest FG, Stewart GG, editors. Handbook of Brewing. 2 ed. Boca Raton: Taylor & Francis Group; 2006. p. 181-285.
38. Čermák P, Palečková V, Houška M, Strohalm J, Novotná P, Mikyška A, et al. Inhibitory effects of fresh hops on Helicobacter pylori strains. Czech Journal of Food Sciences. 2015;33(4):302-7.

39. Karabín M, Hudcová T, Jelínek L, Dostálek P. Biologically Active Compounds from Hops and Prospects for Their Use. Comprehensive Reviews in Food Science and Food Safety. 2016;15(3):542-67.
40. Chadwick LR, Pauli GF, Farnsworth NR. The pharmacognosy of Humulus lupulus L. (hops) with an emphasis on estrogenic properties. Phytomedicine. 2006;13(1-2):119-31.
41. Zanoli P, Zavatti M. Pharmacognostic and pharmacological profile of Humulus lupulus L. Journal of Ethnopharmacology. 2008;116(3):383-96.
42. Briggs DE, Boulton Ca, Brookes Pa, Steven R. Brewing: science and practice. Cambridge: Woodhead Publishing Limited; 2004. 881- p.
43. Kowaka M, Kokubo E. Composition of Bitter Substances of Hops and Characteristics of Beer Bitterness. American Society of Brewing Chemists. 1976;35:16-21.
44. Clark SM, Vaitheeswaran V, Ambrose SJ, Purves RW, Page JE. Transcriptome analysis of bitter acid biosynthesis and precursor pathways in hop (Humulus lupulus). BMC plant biology. 2013;13(1):12-.
45. Urban J, Dahlberg CJ, Carroll BJ, Kaminsky W. Absolute configuration of beer's bitter compounds. Angewandte Chemie - International Edition. 2013;52(5):1553-5.
46. Cook AH, Harris G. The chemistry of hop constituents. Part I. Humulinone, a new constituent of hops. Journal of the Chemical Society. 1950;0:1873-6.
47. Spetsig LO, Steninger M. Hulupones, a new group of hop bitter substances. J I Brewing. 1960;66(5):413-7.
48. Algazzali V, Shellhammer T. Bitterness Intensity of Oxidized Hop Acids: Humulinones and Hulupones. Journal of the American Society for Brewing Chemists. 2016;74(1):36-43.
49. Oladokun O, Tarrega A, James S, Smart K, Hort J, Cook D. The impact of hop bitter acid and polyphenol profiles on the perceived bitterness of beer. Food Chemistry. 2016;205:212-20.
50. Henley T, Reddivari L, Broeckling CD, Bunning M, Miller J, Avens JS, et al. American India Pale Ale matrix rich in xanthohumol is potent in suppressing proliferation and elevating apoptosis of human colon cancer cells. International Journal of Food Science and Technology. 2014;49(11):2464-71.
51. Machado JC, Faria MA, Melo A, Ferreira IMPLVO. Antiproliferative effect of beer and hop compounds against human colorectal adenocarcinome Caco-2 cells. J Funct Foods. 2017;36:255-61.
52. Milligan SR, Kalita JC, Heyerick A, Rong H, De Cooman L, De Keukeleire D. Identification of a potent phytoestrogen in hops (Humulus lupulus L.) and beer. Journal of Clinical Endocrinology and Metabolism. 1999;84(6):2249-52.
53. Schaefer O, Hümpel M, Fritzemeier K-H, Bohlmann R, Schleuning W-D. 8-Prenyl naringenin is a potent ERα selective phytoestrogen present in hops and beer. The Journal of Steroid Biochemistry and Molecular Biology. 2003;84(2):359-60.
54. Stevens JF, Page JE. Xanthohumol and related prenylflavonoids from hops and beer: to your good health! Phytochemistry. 2004;65(10):1317-30.
55. Dresel M, Dunkel A, Hofmann T. Sensomics Analysis of Key Bitter Compounds in the Hard Resin of Hops (Humulus lupulus L.) and Their Contribution to the Bitter Profile of Pilsner-Type Beer. Journal of Agricultural and Food Chemistry. 2015;63(13):3402-18.
56. Almaguer C, Gastl M, Arendt EK, Becker T. Contributions of hop hard resins to beer quality. BrewingScience. 2012;65(7-8):118-29.
57. Moir M. Hops: A millennium review. J Am Soc Brew Chem. 2000;58(4):131-46.
58. Gonçalves JL, Figueira Ja, Rodrigues FP, Ornelas LP, Branco RN, Silva CL, et al. A powerful methodological approach combining headspace solid phase microextraction, mass

spectrometry and multivariate analysis for profiling the volatile metabolomic pattern of beer starting raw materials. Food Chem. 2014;160:266-80.

59. Liu Z, Wang L, Liu Y. Analyzing Differences in Freshness of SA-1 Hops by Headspace Solid-Phase Microextraction Gas Chromatography – Mass Spectrometry Combined with Chemometrics. J Am Soc Brew Chem. 2017;75(3):193-200.
60. Steinhaus M, Schieberle P. Comparison of the most odor-active compounds in fresh and dried hop cones (Humulus lupulus L. variety spalter select) based on GC-olfactometry and odor dilution techniques. J Agric Food Chem. 2000;48(5):1776-83.
61. Eyres GT, Marriott PJ, Dufour JP. Comparison of odor-active compounds in the spicy fraction of hop (Humulus lupulus L.) essential oil from four different varieties. J Agric Food Chem. 2007;55(15):6252-61.
62. Van Opstaele F, De Causmaecker B, Aerts G, De Cooman L. Characterization of novel varietal floral hop aromas by headspace solid phase microextraction and gas chromatography-mass spectrometry/olfactometry. J Agric Food Chem. 2012;60(50):12270-81.
63. Peacock VE, Deinzer ML. Chemistry of Hop Aroma in Beer. J Am Soc Brew Chem. 1981;39(4):136-41.
64. Lermusieau G, Bulens M, Collin S. Use of GC-olfactometry to identify the hop aromatic compounds in beer. J Agric Food Chem. 2001;49(8):3867-74.
65. Takoi K, Itoga Y, Koie K, Kosugi T, Shimase M, Katayama Y, et al. The Contribution of Geraniol Metabolism to the Citrus Flavour of Beer: Synergy of Geraniol and β-Citronellol Under Coexistence with Excess Linalool. J I Brewing. 2010;116(3):251-60.
66. Goiris K, Ridder M, Rouck G, Boeykens A, Opstaele F, Aerts G, et al. The Oxygenated Sesquiterpenoid Fraction of Hops in Relation to the Spicy Hop Character of Beer. J I Brewing. 2002;108(1):86-93.
67. Dresel M, Praet T, Van Opstaele F, Van Holle A, Naudts D, De Keukeleire D, et al. Comparison of the analytical profiles of volatiles in single-hopped worts and beers as a function of the hop variety. BrewingScience. 2015;68(1-2):8-28.
68. Schnaitter M, Kell A, Kollmannsberger H, Schüll F, Gastl M, Becker T. Scale-up of Dry Hopping Trials: Importance of Scale for Aroma and Taste Perceptions. Chem Ing Tech. 2016;88(12):1955-65.
69. IHGC, editor IHGC Hop variety list. IHGC meetings; 2018 12 November 2018; Nuremberg: The International Hop Growers' Convention.
70. Morado R. Larousse da Cerveja. 1 ed. São Paulo: Lafonte; 2009. 360- p.
71. Brewers Association, editor Beer Style Guidelines2017; Boulder, CO.
72. Martins A, editor Um futuro para a produção de lúpulo em Portugal. I Jornada de Lúpulos; 2015; Bragança: Instituto Politécnico de Bragança.
73. Rodrigues MÂ, Sá Morais J, editors. A cultura do lúpulo em Bragança. Aspetos agronómicos inovadores e potencial e expansão. I Jornada de lúpulo; 2015 2015; Bragança: Instituto Politécnico de Bragança.
74. Masschelein CA. The biochemistry of maturation. J I Brewing. 1986;92(3):213-9.
75. Statista. Craft Beer Industry in the U.S. - Statistics & Facts statista.com: Statista; 2016 [Available from: https://www.statista.com/statistics/687621/dollar-sales-leading-craft-beer-styles-us/.
76. BJCP, editor Beer Style Guidelines2015: Beer Judge Certification Program.
77. Statista. Beer production worldwide from 1998 to 2017 (in billion hectoliters) The Statistics Portal: Statista.com; 2019 [Available from: https://www.statista.com/statistics/270275/worldwide-beer-production/.
78. CraftBeer.com. What is Craft Beer? : Brewers Association; 2017 [Available from: https://www.craftbeer.com/breweries/what-is-a-craft-brewery.
79. Watson B, Herz J. National Beer Sales & Production Data. The New Brewer. 2018.
80. The Brewers of Europe. Beer statistics. Brussels: The Brewers of Europe; 2018 December 2018. Report No.: 978-2-9601382-7-6.

81. Alvarenga D. Número de cervejarias no Brasil quase dobra em 3 anos e setor volta criar empregos g1.globo.com: Globo.com; 2018 [Available from: https://g1.globo.com/economia/pme/noticia/numero-de-cervejarias-no-brasil-quase-dobra-em-3-anos-e-setor-volta-criar-empregos.ghtml.
82. IHGC. Economic comission - Summary reports. http://www.hmelj-giz.si: The International Hop Growers' Convention (IHGC); 2018 12 November 2018.
83. IHGC. Economic comission - Summary reports. http://www.hmelj-giz.si: The International Hop Growers' Convention (IHGC); 2009 - 2018 12 November 2018.
84. Barth-Haas G. The Barth Report. Nuremberg; 2012.
85. Verband Deutscher Hopfenpflanzer eV. Hopfenmarktbericht Nr. 8 vom 20. November 2018. www.deutscher-hopfen: Verband Deutscher Hopfenpflanzer e.V.; 2018.
86. NASS. National Hop Report: 2018 Hop Production Up 1 Percent From Last Year. United States Department of Agriculture (USDA): National Agricultural Statistics Service (NASS); 2018 19 December 2018.
87. Compare data [Internet]. Food and Agriculture Organization of the United State. 2019 [cited 23 April 2019]. Available from: http://www.fao.org/faostat/en/#home.
88. Malířová I. Hop growing in the newly established czechoslovak republic. Czech Hops. 2018:4-7.
89. IHGC. Economic comission - Summary reports. http://www.hmelj-giz.si: The International Hop Growers' Convention (IHGC); 2014 10 November 2014.
90. Association BH. www.britishhops.org.uk/: The British Hop Association; 2019 [Available from: https://www.britishhops.org.uk/hop-breeding/.
91. IHGC. Economic comission - Summary reports. http://www.hmelj-giz.si: The International Hop Growers' Convention (IHGC); 2017 10 November 2017.
92. Donelan D. 2018 Harvest info nzhops.co.nz: New Zealand Hops Limited; 2018 [Available from: https://www.nzhops.co.nz/2018-harvest-info.
93. Hopsteiner. Las nuevas variedades de lúpulo ocuparán unas 40 hectáreas Hopsteiner España: https://hopsteiner.es/ver-noticia.php?id=17; 2019 [Available from: https://hopsteiner.es/ver-noticia.php?id=17.
94. Hops from alsace the new fragrances [press release]. www.hops-comptoir.com/: Comptoir agricole2015.
95. Barth-Hass G. The Barth Report. Nuremberg; 2015.
96. ASBC Methods, editor Hops 14: α-acids and β-acids in hops and hop extracts by HPLC (international method)2008; St. Paul, MN, USA.
97. EBC Analytica. EBC 7.7: Alpha and beta acids in hops and hops product by HPLC. Brussels; 2012.
98. ASBC Methods, editor Hops 6: α- and β-acids in hops and hop pellets by spectrophotometry and by conductometric titration2008; St. Paul, MN, USA.
99. ASBC Methods, editor Beer Bitterness. Subcommittee on Determination of Isohumulones in Beer; 1968: American Society of Brewing Chemists.
100. EBC Analytica, editor EBC 9.8 - Bitterness of Beer (IM)2004; Brussels. BrewUp: The Brewers of Europe.
101. ASBC Methods, editor Hops 12: Hop Storage Index (HSI)2008; St. Paul, MN, USA.
102. EBC Analytica, editor EBC 7.14: Total Polyphenols in Hops and Hops Pellets2015; Brussels. BrewUp: The Brewers of Europe.
103. EBC Analytica, editor EBC 9.11 - Total Polyphenols in Beer by Spectrophotometry (IM)2002; Brussels. BrewUp: The Brewers of Europe.

104. Magalhães PJ, Guido LF, Cruz JM, Barros AA. Analysis of xanthohumol and isoxanthohumol in different hop products by liquid chromatography-diode array detection-electrospray ionization tandem mass spectrometry. Journal of Chromatography A. 2007;1150(1-2):295-301.
105. Intelmann D, Haseleu G, Hofmann T. LC-MS/MS quantitation of hop-derived bitter compounds in beer using the ECHO technique. Journal of Agricultural and Food Chemistry. 2009;57(4):1172-82.
106. Kao TH, Wu GY. Simultaneous determination of prenylflavonoid and hop bitter acid in beer lee by HPLC-DAD-MS. Food Chemistry. 2013;141(2):1218-26.
107. Chapman AC. The Essential Oil of Hops. J I Brewing. 1929;35(3):247-55.
108. Chapman AC. The Essential Oil of Hops. Journal of the Federated Institutes of Brewing. 1898;4(1):224-35.
109. Chapman AC. The hop and its constituents. A monograph on the hop plant. London: The Brewing trade review; 1905.
110. Buttery RG, Black DR, P. Kealy M. Volatile oxygenated constituents of hops: Identification by combined gas chromatography and mass spectrometry. Journal of Chromatography A. 1965;18:399-402.
111. Kovačevič M, Kač M. Solid-phase microextraction of hop volatiles. Potential use for determination and verification of hop varieties. J Chromatogr A. 2001;918(1):159-67.
112. Jorge K, Trugo LC. Discrimination of different hop varieties using headspace gas chromatographic data. J Braz Chem Soc. 2003;14(3):411-5.
113. EBC Analytica. EBC 7.10: Hop oil content of hops and hops products. Brussels; 2002.
114. ASBC Methods, editor Hops 13: Total Essential Oils in Hops and Hop Pellets by Steam Distillation2008; St. Paul, MN, USA.
115. Field JA, Nickerson G, James DD, Heider C. Determination of Essential Oils in Hops by Headspace Solid-Phase Microextraction. Journal of Agricultural and Food Chemistry. 1996;44:1768-72.
116. Zapata J, Mateo-Vivaracho L, Lopez R, Ferreira V. Automated and quantitative headspace in-tube extraction for the accurate determination of highly volatile compounds from wines and beers. Journal of Chromatography A. 2012;1230:1-7.
117. Socaci SA, Socaciu C, Tofană M, Raţi IV, Pintea A. In-tube extraction and GC-MS analysis of volatile components from wild and cultivated sea buckthorn (Hippophae rhamnoides L. ssp. Carpatica) berry varieties and juice. Phytochemical Analysis. 2013;24(4):319-28.
118. Michiu D, Socaci SA, Jimborean MA, Mudura E, Fărcaş AC, Biriş-Dorhoi SE, et al. Determination of Volatile Markers from Magnum Hops in Beer by In-Tube Extraction—Gas Chromatography—Mass Spectrometry. Analytical Letters. 2018;51(18):2967-80.
119. Liu Z, Wang L, Liu Y. Rapid differentiation of Chinese hop varieties (Humulus lupulus) using volatile fingerprinting by HS-SPME-GC–MS combined with multivariate statistical analysis. Journal of the Science of Food and Agriculture. 2018;98(10):3758-66.
120. Maule DR. Rapid gas chromatographic examination of beer flavour. J I Brewing. 1967;73(4):351-61.
121. Neiens SD, Steinhaus M. Odor-Active Compounds in the Special Flavor Hops Huell Melon and Polaris. J Agric Food Chem. 2018;66(6):1452-60.
122. Kishimoto T, Wanikawa A, Kono K, Shibata K. Comparison of the odor-active compounds in unhopped beer and beers hopped with different hop varieties. J Agric Food Chem. 2006;54(23):8855-61.
123. Wiesen E, Drexler G, Hinz S, Insa AM, Algazzali V, Kostelecky T, et al. The language of hops - How to implement and applay common discriptioin of hop flavours. European Brewery Convention; Ljubljana: Barth-Haas Group; 2017.
124. Stucky GJ, McDaniel MR. Raw Hop Aroma Qualities by Trained Panel Free-Choice Profiling. J Am Soc Brew Chem. 1997;55(2):65-72.

125. Koetter U, Biendl M. Hops (Humulus lupulus): A Review of its Historic and Medicinal Uses. The Journal of American Botanical Council. 2010:44-57.
126. Tagashira M, Watanabe M, Uemitsu N. Antioxidative Activity of Hop Bitter Acids and Their Analogues. Biosci Biotechnol Biochem. 1995;59(4):740-2.
127. Hudcová T, Bryndová J, Fialová K, Fiala J, Karabín M, Jelínek L, et al. Antiproliferative effects of prenylflavonoids from hops on human colon cancer cell lines. Institute of Brewing & Distilling. 2014;120:225-30.
128. Maye JP, Smith R, Leker J. Humulinone Formation in Hops and Hop Pellets and Its Implications for Dry Hopped Beers. Technical Quarterly. 2016;53(1):23-7.
129. Taniguchi Y, Matsukura Y, Ozaki H, Nishimura K, Shindo K. Identification and Quantification of the Oxidation Products Derived from αlpha Acids and beta Acids During Storage of Hops (Humulus lupulus L.). Journal of Agricultural and Food Chemistry. 2013;61:3121-30.
130. Taniguchi Y, Matsukura Y, Taniguchi H, Koizumi H, Katayama M. Development of preparative and analytical methods of the hop bitter acid oxide fraction and chemical properties of its components. Bioscience, Biotechnology, and Biochemistry. 2015;79(10):1684-94.
131. Cattoor K, Dresel M, De Bock L, Boussery K, Van Bocxlaer J, Remon JP, et al. Metabolism of hop-derived bitter acids. Journal of Agricultural and Food Chemistry. 2013;61(33):7916-24.
132. Haseleu G, Intelmann D, Hofmann T. Structure determination and sensory evaluation of novel bitter compounds formed from β-acids of hop (Humulus lupulus L.) upon wort boiling. Food Chemistry. 2009;116(1):71-81.
133. Mongelli A, Rodolfi M, Ganino T, Marieschi M, Dall'Asta C, Bruni R. Italian hop germplasm: Characterization of wild Humulus lupulus L. genotypes from Northern Italy by means of phytochemical, morphological traits and multivariate data analysis. Ind Crop Prod. 2015;70:16-27.
134. Gu F, WuJiu Z, HaiFeng L. Identification of hop varieties with AFLP technique. Hop Bull. 2006;13:53-9.
135. Abbott MS, Fedele MJ. A DNA-based varietal identification procedure for hop leaf tissue. J I Brewing. 1994;100(4):283-5.
136. Čerenak A, Jakše J, Javornik B. Identification and Differentiation of Hop Varieties Using Simple Sequence Repeat Markers. J Am Soc Brew Chem. 2004;62(1):1-7.
137. Stajner N, Jakse J, Kozjak P, Javornik B. The isolation and characterisation of microsatellites in hop (Humulus lupulus L.). Plant Science. 2005;168(1):213-21.
138. Jakse J, Luthar Z, Javornik B. New polymorphic dinucleotide and trinucleotide microsatellite loci for hop Humulus lupulus L. Molecular Ecology Resources. 2008;8(4):769-72.
139. Koelling J, Coles MC, Matthews PD, Schwekendiek A. Development of new microsatellite markers (SSRs) for Humulus lupulus. Molecular Breeding. 2012;30(1):479-84.
140. Howard EL, Whittock SP, Jakše J, Carling J, Matthews PD, Probasco G, et al. High-throughput genotyping of hop (Humulus lupulus L.) utilising diversity arrays technology (DArT). Theoretical and Applied Genetics. 2011;122(7):1265-80.
141. Yamauchi H, Mukouzaka Y, Taniguchi T, Nakashima K, Furukubo S, Harada M. Newly developed snp-based identification method of hop varieties. J Am Soc Brew Chem. 2014;72(4):239-45.
142. Matthews P, Coles M, Pitra N. Next generation sequencing for a plant of great tradition: Application of NGS to SNP detection and validation in hops (Humulus lupulus L). Monatsschrift Fur Brauwissenschaft (Internet). 2013;66:186.
143. Patzak J, Nesvadba V, Krofta K, Henychova A, Marzoev AI, Richards K. Evaluation of genetic variability of wild hops (Humulus lupulus L.) in Canada and the Caucasus region by chemical and molecular methods. Genome. 2010;53(7):545-57.

144. Patzak J. Comparison of RAPD, STS, ISSR and AFLP molecular methods used for assessment of genetic diversity in hop (Humulus lupulus L.). Euphytica. 2001;121(1):9-18.
145. Pillay M, Kenny ST. Structure and inheritance of ribosomal DNA variants in cultivated and wild hop, Humulus lupulus L. Theoretical and Applied Genetics. 1996;93(3):333-40.
146. Š Uštar-Vozlič J, Javornik B. Genetic relationships in cultivars of hop, Humulus lupulus L., determined by RAPD analysis. Plant Breeding. 1999;118(2):175-81.
147. Peredo EL, Ángeles Revilla M, Reed BM, Javornik B, Cires E, Prieto JAF, et al. The influence of European and American wild germplasm in hop (Humulus lupulus L.) cultivars. Genet Resour Crop Ev. 2010;57(4):575-86.
148. Rodolfi M, Silvanini A, Chiancone B, Marieschi M, Fabbri A, Bruni R, et al. Identification and genetic structure of wild Italian Humulus lupulus L. and comparison with European and American hop cultivars using nuclear microsatellite markers. Genet Resour Crop Ev. 2018;65(5):1405-22.
149. Jakse J, Stajner N, Kozjak P, Cerenak A, Javornik B. Trinucleotide microsatellite repeat is tightly linked to male sex in hop (Humulus lupulus L.). Molecular Breeding. 2008;21(2):139-48.
150. Leaché AD, Oaks JR. The Utility of Single Nucleotide Polymorphism (SNP) Data in Phylogenetics. Annual Review of Ecology, Evolution, and Systematics. 2017;48(1):69-84.
151. McAdam EL, Freeman JS, Whittock SP, Buck EJ, Jakse J, Cerenak A, et al. Quantitative trait loci in hop (Humulus lupulusL.) reveal complex genetic architecture underlying variation in sex, yield and cone chemistry. BMC Genomics. 2013;14(1):360.
152. Gori A, Cerboneschi M, Tegli S. High-Resolution Melting Analysis as a Powerful Tool to Discriminate and Genotype Pseudomonas savastanoi Pathovars and Strains. Plos One. 2012;7(1):e30199.
153. Słomka M, Sobalska-Kwapis M, Wachulec M, Bartosz G, Strapagiel D. High Resolution Melting (HRM) for High-Throughput Genotyping—Limitations and Caveats in Practical Case Studies. International Journal of Molecular Sciences. 2017;18(11):2316.
154. Wittwer CT, Reed GH, Gundry CN, Vandersteen JG, Pryor RJ. High-Resolution Genotyping by Amplicon Melting Analysis Using LCGreen. Clinical Chemistry. 2003;49(6):853-60.
155. Nickerson GB, Williams PA, Haunold A. Varietal Differences in the Proportions of Cohumulone, Adhumulone, and Humulone in Hops. J Am Soc Brew Chem. 1986;44(2):91-4.
156. De Cooman L, Everaert E, De Keukeleire D. Quantitative analysis of hop acids, essential oils and flavonoids as a clue to the identification of hop varieties. Phytochemical Analysis. 1998;9(3):145-50.
157. Kenny ST. Identification of U.S.-Grown Hop Cultivars by Hop Acid and Essential Oil Analyses. J Am Soc Brew Chem. 1990;48:3-8.
158. Kovačevič M, Kač M. Solid-phase microextraction of hop volatiles: Potential use for determination and verification of hop varieties. Journal of Chromatography A. 2001;918(1):159-67.
159. Kovačevič M, Kač M. Determination and verification of hop varieties by analysis of essential oils. Food Chemistry. 2002;77(4):489-94.
160. Cerenak A, Pavlovic M, Monika OL, Košir IJ. Characterization of slovenian hop (Humulus lupulus L.) varieties by analysis of essential oil. Hop Bull. 2011;18:27-32.
161. Neugrodda C, Gastl M, Becker T. Protein profile characterization of hop (Humulus lupulus L.) varieties. J Am Soc Brew Chem. 2014;72(3):184-91.
162. Ocvirk M, Grdadolnik J, Košir IJ. Determination of the botanical origin of hops (Humulus lupulus L.) using different analytical techniques in combination with statistical methods. J I Brewing. 2016;122(3):452-61.
163. Shellie RA, Poynter SDH, Li J, Gathercole JL, Whittock SP, Koutoulis A. Varietal characterization of hop (Humulus lupulus L.) by GC–MS analysis of hop cone extracts. Journal of Separation Science. 2009;32(21):3720-5.

164. dos Santos CAT, Páscoa RNMJ, Lopes JA. A review on the application of vibrational spectroscopy in the wine industry: From soil to bottle. TrAC Trends in Analytical Chemistry. 2017;88:100-18.
165. Emura K, Yamanaka S, Isoda H, Watanabe KN. Estimation for Different Genotypes of Plants based on DNA Analysis using Near-infrared (NIR) and Fourier-transform Infrared (FT-IR) Spectroscopy. Breeding Science. 2006;56(4):399-403.
166. Gutiérrez S, Tardaguila J, Fernández-Novales J, Diago MP. Data Mining and NIR Spectroscopy in Viticulture: Applications for Plant Phenotyping under Field Conditions. Sensors. 2016;16(2):236.
167. Vaz M, Meirinhos-Soares L, Sousa CCS, Ramirez M, Melo-Cristino J, Lopes JA. Serotype discrimination of encapsulated Streptococcus pneumoniae strains by Fourier-transform infrared spectroscopy and chemometrics. Journal of Microbiological Methods. 2013;93(2):102-7.
168. Barth-Haas G. The Barth Report. Nuremberg; 2017.
169. Naes T, Isaksson T, Fearn T, Davies T. A User Friendly Guide to Multivariate Calibration and Classification. Chichester: NIR publications; 2004.
170. Barker M, Rayens W. Partial least squares for discrimination. Journal of Chemometrics. 2003;17(3):166-73.
171. Páscoa RNMJ, Lopo M, Teixeira dos Santos CA, Graça AR, Lopes JA. Exploratory study on vineyards soil mapping by visible/near-infrared spectroscopy of grapevine leaves. Computers and Electronics in Agriculture. 2016;127:15-25.
172. Murakami A. Hop variety classification using the genetic distance based on RAPD. J I Brewing. 2000;106(3):157-61.
173. Henning JA, Townsend MS, Kenny S. Potential heterotic crosses in hops as estimated by AFLP-based genetic diversity and coefficient of coancestry. J Am Soc Brew Chem. 2004;62(2):63-70.
174. Eyres G, Dufour J-P. 22 - Hop Essential Oil: Analysis, Chemical Composition and Odor Characteristics. In: Preedy VR, editor. Beer in Health and Disease Prevention. San Diego: Academic Press; 2009. p. 239-54.
175. Roberts MT, Dufour J-P, Lewis AC. Application of comprehensive multidimensional gas chromatography combined with time-of-flight mass spectrometry (GC×GC-TOFMS) for high resolution analysis of hop essential oil. JSS. 2004;27(5-6):473-8.
176. Vázquez-Araújo L, Rodríguez-Solana R, Cortés-Diéguez SM, Domínguez JM. Use of hydrodistillation and headspace solid-phase microextraction to characterize the volatile composition of different hop cultivars. J Sci Food Agric. 2013;93(10):2568-74.
177. Gonçalves J, Figueira J, Rodrigues F, Câmara JS. Headspace solid-phase microextraction combined with mass spectrometry as a powerful analytical tool for profiling the terpenoid metabolomic pattern of hop-essential oil derived from Saaz variety. JSS. 2012;35(17):2282-96.
178. MacFie HJ, Bratchell N, Greenhoff K, Vallis LV. Designs to balance the effect of order of presentation and first-order carry-over effects in hall tests. J Sens Stud. 1989;4(2):129-48.
179. Urruty L, Giraudel J-L, Lek S, Roudeillac P, Montury M. Assessment of Strawberry Aroma through SPME/GC and ANN Methods. Classification and Discrimination of Varieties. J Agric Food Chem. 2002;50(11):3129-36.
180. Liland KH. Multivariate methods in metabolomics – from pre-processing to dimension reduction and statistical analysis. Trends Anal Chem. 2011;30(6):827-41.
181. Sugimoto M, Kawakami M, Robert M, Soga T, Tomita M. Bioinformatics Tools for Mass Spectroscopy-Based Metabolomic Data Processing and Analysis. Curr Bioinform. 2012;7(1):96-108.

182. Cochran WG. The comparison of percentages in matched samples. Biometrika. 1950;37(3-4):256-66.
183. Warnes GR, Bolker B, Bonebakker L, Gentleman R, Liaw WHA, Lumley T, et al. Various R Programming Tools for Plotting Data. R Package Version 3.0.1.1. The Comprehensive R Archive Network: The Comprehensive R Archive Network; 2019 [updated March 13, 2019. 2019/01/27:[68]. Available from: https://cran.r-project.org/web/packages/gplots/index.html.
184. Kassambara A. Factoextra: Extract and Visualize the Results of PCA, CA and MCA. R Package Version 1.0.2. Retrieved from https://cran.r-project.org/web/packages/factoextra/index.html. 2015.
185. Husson F, Josse J, Le S, Mazet J. FactoMineR: Multivariate Exploratory Data anslysis and Data Mining. R package version 1.29 2015 [Available from: http://cran.r-project.org/package=FactoMineR.
186. Gros J, Nizet S, Collin S. Occurrence of odorant polyfunctional thiols in the super alpha Tomahawk hop cultivar. Comparison with the thiol-rich Nelson Sauvin bitter variety. J Agric Food Chem. 2011;59:8853-65.
187. Van Opstaele F, Praet T, Aerts G, De Cooman L. Characterization of novel single-variety oxygenated sesquiterpenoid hop oil fractions via headspace solid-phase microextraction and gas chromatography-mass spectrometry/olfactometry. J Agric Food Chem. 2013;61(44):10555-64.
188. Luebke W. The Good Scents Company http://www.thegoodscentscompany.com: The Good Scents Company; 2018 [Available from: http://www.thegoodscentscompany.com.
189. Ballabio D, Consonni V. Classification tools in chemistry. Part 1: linear models. PLS-DA. Anal Methods. 2013;5(16):3790-8.
190. Gromski PS, Muhamadali H, Ellis DI, Xu Y, Correa E, Turner ML, et al. A tutorial review: Metabolomics and partial least squares-discriminant analysis – a marriage of convenience or a shotgun wedding. Anal Chim Acta. 2015;879:10-23.
191. Szymańska E, Saccenti E, Smilde AK, Westerhuis JA. Double-check: validation of diagnostic statistics for PLS-DA models in metabolomics studies. Metabolomics. 2012;8(1):3-16.
192. Davis J, Goadrich M. The relationship between Precision-Recall and ROC curves. Proceedings of the 23rd international conference on Machine learning; Pittsburgh, Pennsylvania, USA. 1143874: ACM; 2006. p. 233-40.
193. Mehmood T, Liland KH, Snipen L, Sæbø S. A review of variable selection methods in Partial Least Squares Regression. Chemom Intell Lab Syst. 2012;118:62-9.
194. Steingass CB, Jutzi M, Müller J, Carle R, Schmarr H-G. Ripening-dependent metabolic changes in the volatiles of pineapple (Ananas comosus (L.) Merr.) fruit: II. Multivariate statistical profiling of pineapple aroma compounds based on comprehensive two-dimensional gas chromatography-mass spectrometry. Anal Bioanal Chem. 2015;407(9):2609-24.
195. Lermusieau G, Collin S. Varietal Discrimination of Hop Pellets . II. Comparison Between Fresh and Aged Samples. J Am Soc Brew Chem. 2001;59(1):39-43.
196. Perpete P, Melotte L, Dupire S, Collin S. Varietal discrimination of hop pellets by essential oil analysis I. Comparison of fresh samples. J Am Soc Brew Chem. 1998;56(3):104-8.
197. Jelinek L, Doleckova M, Karabin M, Hudcova T, Kotlikova B, Dostalek P. Influence of Growing Area, Plant Age, and Virus Infection on the Contents of Hop Secondary Metabolites. Czech J Food Sci. 2012;30(6):541-7.
198. Gahr A, Forster A. A Comparison of the Analytical and Brewing Chracteristics of Cascade and Comet Hop Varieties as Grown in Yakima (USA) and Hallertau (Germany). BrSc. 2014;67:137-48.
199. Varela P, Ares G. Sensory profiling, the blurred line between sensory and consumer science. A review of novel methods for product characterization. Food Res Int. 2012;48(2):893-908.

200. Parente ME, Manzoni AV, Ares G. External preference mapping of commercial antiaging creams based on consumers' responses to a check-all-that-apply question. J Sens Stud. 2011;26(2):158-66.
201. Nokels L, Fahmy T, Crochemore S. Interpretation of the Preferences of Automotive Customers Applied to Air Conditioning Supports by Combining GPA and PLS Regression. In: Esposito Vinzi V, Chin WW, Henseler J, Wang H, editors. Handbook of Partial Least Squares: Concepts, Methods and Applications. Berlin, Heidelberg: Springer Berlin Heidelberg; 2010. p. 775-89.
202. Luebke W. The Good Scents Company: 2-undecanone http://www.thegoodscentscompany.com/data/rw1021151.html#tonotes: The Good Scents Company; 1983 [Available from: http://www.thegoodscentscompany.com/data/rw1021151.html#tonotes.
203. Mosciano G. Perfumer & Flavorist: 2-Undecanone Natural https://www.perfumerflavorist.com/flavor/library/?MATCHAGAINSTname=undecanone&detail=y&id=6246: Allured Business Media; 1991 [Available from: https://www.perfumerflavorist.com/flavor/library/?MATCHAGAINSTname=undecanone&detail=y&id=6246.
204. Hanke S, Schull F, Seigner E, Lutz A. Development of a Tasting Scheme and a New Systematic Evaluation Program for new German Breeding Lines by example of the New German varieties Callista (CI) and Ariana (AN). Brewing Science. 2016;69(11-12):94-102.
205. Forster A, Gahr A. On the Fate of Certain Hop Substances during Dry Hopping Brewing Science. 2013;66:93-103.
206. Lafontaine SR, Shellhammer TH. Sensory Directed Mixture Study of Beers Dry-Hopped with Cascade, Centennial, and Chinook. J Am Soc Brew Chem. 2018;76(3):199-208.
207. Lermusieau G, Collin S. Volatile Sulfur Compounds in Hops and Residual Concentrations in Beer - A Review. J Am Soc Brew Chem. 2003;61(3):109-13.
208. Du X, Whitaker V, Rouseff R. Changes in strawberry volatile sulfur compounds due to genotype, fruit maturity and sample preparation. Flavour and Fragrance Journal. 2012;27(6):398-404.
209. Engel K-H. The Importance of Sulfur-Containing Compounds to Fruit Flavors. In: Teranishi R, Wick EL, Hornstein I, editors. Flavor Chemistry: Thirty Years of Progress. Boston, MA: Springer US; 1999. p. 265-73.
210. Tapp EJ, Cummins I, Brassington D, Edwards R. Determination and Isolation of a Thioesterase from Passion Fruit (Passiflora edulis Sims) That Hydrolyzes Volatile Thioesters. Journal of Agricultural and Food Chemistry. 2008;56(15):6623-30.
211. Peacock VE, Deinzer ML, Likens ST, Nickerson GB, McGill LA. Floral hop aroma in beer. Journal of Agricultural and Food Chemistry. 1981;29(6):1265-9.
212. Lam KC, Foster RT, Deinzer ML. Aging of hops and their contribution to beer flavor. Journal of Agricultural and Food Chemistry. 1986;34(4):763-70.
213. Analysis AMo. Hop Flavor Database. In: Database HF, editor.: American Society of Brewing Chemists.
214. Takoi K, Degueil M, Shinkaruk S, Thibon C, Kurihara T, Toyoshima K, et al. Specific flavor compounds derived from nelson sauvin hop and synergy of these compounds. BrewingScience. 2009;62(7-8):108-18.
215. Seigner E, Lutz A, Kneidl J, Kammhuber K, editors. Breeding of Special Flavor Hops to pave the way to the craft brewers. International Hop Growers` Convention; 2013 04 - 09 JUNE 2013; Ukraine: Scientific Commission of International Hop Growers` Convention (I.H.G.C.); 2013.
216. Schnaitter M, Wimmer A, Kollmannsberger H, Gastl M, Becker T. Influence of hop harvest date of the 'Mandarina Bavaria' hop variety on the sensory evaluation of dry-hopped top-fermented beer. J I Brewing. 2016;122(4):661-9.

217. Miranda CL, Elias VD, Hay JJ, Choi J, Reed RL, Stevens JF. Xanthohumol improves dysfunctional glucose and lipid metabolism in diet-induced obese C57BL/6J mice. Arch Biochem Biophys. 2016;599:22-30.
218. Jeong HM, Han EH, Jin YH, Choi YH, Lee KY, Jeong HG. Xanthohumol from the hop plant stimulates osteoblast differentiation by RUNX2 activation. Biochem Bioph Res Co. 2011;409(1):82-9.
219. Almaguer C, Gastl M, Arendt EK, Becker T. Comparative Study of the Contribution of Hop (Humulus lupulus L.) Hard Resins Extracted from Different Hop Varieties to Beer Quality Parameters. J Am Soc Brew Chem. 2015;73(2):115-23.
220. Schnaitter M, Wimmer A, Kollmannsberger H, Gastl M, Becker T. Influence of hop harvest date of the 'Mandarina Bavaria' hop variety on the sensory evaluation of dry-hopped top-fermented beer. J I Brewing. 2016;122(4):661-9.
221. Karabin M, Jelinek L, Kincl T, Hudcova T, Kotlikova B, Dostalek P. New approach to the production of xanthohumol-enriched beers. J I Brewing. 2013;119(3):98-102.
222. Cocuzza S, Mitter W. Dry hopping - a study of various parameters. Brew Beverage Ind Int. 2013;4:70–4.
223. Beer-23 Beer Bitterness: Iso-α-acids by solid-phase extraction and HPLC, (1993).
224. John I. Haas I. Barth-Haas hops companion: A guide to the varieties of hops and hop products. Washington DC, USA: John I. Haas, Inc.; 2016.
225. Skinner RN, Kavanagh TE, Clarke BJ. Stability of Resins in Pelletized Hops. J I Brewing. 1979;85(1):7-10.
226. Kaneda H, Tsuchiya Y, Kano Y, Kamimura M, Osawa T, Kawakishi S. Evaluation of Storage Stability of Hop Pellets by Chemiluminescence Analysis. J I Brewing. 1990;96(6):395-8.
227. Sharp DC, Qian YP, Shellhammer G, Shellhammer TH. Contributions of Select Hopping Regimes to the Terpenoid Content and Hop Aroma Profile of Ale and Lager Beers. J Am Soc Brew Chem. 2017;75(2):93-100.
228. Parkin E, Shellhammer T. Toward Understanding the Bitterness of Dry-Hopped Beer. J Am Soc Brew Chem. 2017;75(4):363-8.
229. Cibaka MLK, Decourriere L, Lorenzo-Alonso CJ, Bodart E, Robiette R, Collin S. 3-Sulfanyl-4-methylpentan-1-ol in Dry-Hopped Beers: First Evidence of Glutathione S-Conjugates in Hop (Humulus lupulus L.). J Agr Food Chem. 2016;64(45):8572-82.
230. Oladokun O, Tarrega A, James S, Smart K, Hort J, Cook D. The impact of hop bitter acid and polyphenol profiles on the perceived bitterness of beer. Food Chem. 2016;205:212-20.
231. Stevens JF, Taylor AW, Deinzer ML. Quantitative analysis of xanthohumol and related prenylflavonoids in hops and beer by liquid chromatography tandem mass spectrometry. Journal of Chromatography A. 1999;832(1-2):97-107.
232. Biendl M, Methner FJ, Stettner G, Walker C. Brewing trials with a xanthohumol-enriched hop product. Brauwelt International. 2004;22(3):182-4.
233. Walker CJ, Lence CF, Biendl M. Studies on xanthohumol levels in stout/porter beer. Brauwelt Int. 2003;143:1709-12.
234. Algazzali V, Shellhammer T. Bitterness Intensity of Oxidized Hop Acids: Humulinones and Hulupones. J Am Soc Brew Chem. 2016;74(1):36-43.
235. Wunderlich S, Zurcher A, Back W. Enrichment of xanthohumol in the brewing process. Mol Nutr Food Res. 2005;49(9):874-81.
236. Cattoor KO, Bracke M, Deforce D, De Keukeleire D, Heyerick A. Transport of hop bitter acids across intestinal Caco-2 cell monolayers. Journal of Agricultural and Food Chemistry. 2010;58:4132-40.
237. Van Kuppeveld FJM, Van der Logt JTM, Angulo AF, Van Zoest MJ, Quint WGV, Niesters HGM, et al. Genus- and species-specific identification of mycoplasmas by 16S rRNA amplification. Applied and Environmental Microbiology. 1992;58(8):2606-15.

238. Wunderlich S, Zürcher A, Back W. Enrichment of xanthohumol in the brewing process. Molecular Nutrition and Food Research. 2005;49:874-81.
239. Pan L, Becker H, Gerhäuser C. Xanthohumol induces apoptosis in cultured 40-16 human colon cancer cells by activation of the death receptor- and mitochondrial pathway. Molecular nutrition & food research. 2005;49:837-43.
240. Hadjiolov N, Frank N. Xanthohumol and Sulforaphane induce apoptosis and inhibit proliferation of HT29 and HCT 116 colon cancer cells. Comptes Rendus de L'Academie Bulgare des Sciences. 2009;62(9):1175-82.
241. Miranda CL, Stevens JF, Helmrich A, Henderson MC, Rodriguez RJ, Yang YH, et al. Antiproliferative and cytotoxic effects of prenylated flavonoids from hops (Humulus lupulus) in human cancer cell lines. Food and Chemical Toxicology. 1999;37:271-85.
242. Pang Y, Nikolic D, Zhu D, Chadwick LR, Pauli GF, Farnsworth NR, et al. Binding of the hop (Humulus lupulus L.) chalcone xanthohumol to cytosolic proteins in Caco-2 intestinal epithelial cells. Molecular Nutrition and Food Research. 2007;51:872-9.
243. Engle MJ, Goetz GS, Alpers DH. Caco-2 cells express a combination of colonocyte and enterocyte phenotypes. Journal of Cellular Physiology. 1998;174(3):362-9.
244. Monteiro R, Calhau C, Silva AOE, Pinheiro-Silva S, Guerreiro S, Gärtner F, et al. Xanthohumol inhibits inflammatory factor production and angiogenesis in breast cancer xenografts. Journal of Cellular Biochemistry. 2008;104(5):1699-707.
245. Gerhauser C, Alt A, Heiss E, Gamal-Eldeen A, Klimo K, Knauft J, et al. Cancer Chemopreventive Activity of Xanthohumol, a Natural Product Derived from Hop 1. Molecular Cancer Therapeutics. 2002;1:959-69.
246. Deeb D, Gao X, Jiang H, Arbab AS, Dulchavsky SA, Gautam SC. Growth inhibitory and apoptosis-inducing effects of xanthohumol, a prenylated chalone present in hops, in human prostate cancer cells. Anticancer Research. 2010;30(9):3333-9.
247. Delmulle L, Bellahcène A, Dhooge W, Comhaire F, Roelens F, Huvaere K, et al. Anti-proliferative properties of prenylated flavonoids from hops (Humulus lupulus L.) in human prostate cancer cell lines. Phytomedicine. 2006;13:732-4.
248. Delmulle L, Vanden Berghe T, De Keukeleire D, Vandenabeele P. Treatment of PC-3 and DU145 prostate cancer cells by prenylflavonoids from hop (Humulus lupulus L.) induces a caspase-independent form of cell death. Phytotherapy Research. 2008;22:197-203.
249. Dorn C, Weiss TS, Heilmann J, Hellerbrand C. Xanthohumol, a prenylated chalcone derived from hops, inhibits proliferation, migration and interleukin-8 expression of hepatocellular carcinoma cells. International Journal of Oncology. 2010;36(2):435-41.
250. Lust S, Vanhoecke B, Janssens A, Philippe J, Bracke M, Offner F. Xanthohumol kills B-chronic lymphocytic leukemia cells by an apoptotic mechanism. Molecular Nutrition and Food Research. 2005;49:844-50.
251. Jiang W, Zhao S, Xu L, Lu Y, Lu Z, Chen C, et al. The inhibitory effects of xanthohumol, a prenylated chalcone derived from hops, on cell growth and tumorigenesis in human pancreatic cancer. Biomedicine and Pharmacotherapy. 2015;73:40-7.
252. Sławinska-Brych A, Król SK, Dmoszynska-Graniczka M, Zdzisinska B, Stepulak A, Gagos M. Xanthohumol inhibits cell cycle progression and proliferation of larynx cancer cells in vitro. Chem Biol Interact. 2015;240:110-8.
253. Goto K, Asai T, Hara S, Namatame I, Tomoda H, Ikemoto M, et al. Enhanced antitumor activity of xanthohumol, a diacylglycerol acyltransferase inhibitor, under hypoxia. Cancer Letters. 2005;219:215-22.

254. Chen WJ, Lin JK. Mechanisms of Cancer Chemoprevention by Hop Bitter Acids (Beer Aroma) through Induction of Apoptosis Mediated by Fas and Caspase Cascades. Journal of Agricultural and Food Chemistry. 2004;52:55-64.
255. Saugspier M, Dorn C, Czech B, Gehrig M, Heilmann J, Hellerbrand C. Hop bitter acids inhibit tumorigenicity of hepatocellular carcinoma cells in vitro. Oncology Reports. 2012;28:1423-8.
256. Farag MA, Wessjohann LA. Cytotoxic effect of commercial Humulus lupulus L. (hop) preparations - In comparison to its metabolomic fingerprint. Journal of Advanced Research. 2013;4:417-21.
257. Honma Y, Tobe H, Makishima M, Yokoyama A, Okabe-Kado J. Induction of differentiation of myelogenous leukemia cells by humulone, a bitter in the hop. Leukemia Research. 1998;22:605-10.
258. Lamy V, Roussi S, Chaabi M, Goss F, Schall N, Lobstein A, et al. Chemopreventive effects of lupulone, a hop ??-acid, on human colon cancer-derived metastatic SW620 cells and in a rat model of colon carcinogenesis. Carcinogenesis. 2007;28(7):1575-81.
259. Lamy V, Roussi S, Chaabi M, Gossé F, Lobstein A, Raul F. Lupulone, a hop bitter acid, activates different death pathways involving apoptotic TRAIL-receptors, in human colon tumor cells and in their derived metastatic cells. Apoptosis. 2008;13:1232-42.
260. Liu CB, Chen L-H, Cheng A-C, Chen W-J, Tsai M-L, Liu Y, et al. Hexahydro-β-acids induce apoptosis through mitochondrial pathway, GADD153 expression, and caspase activation in human leukemia cells. Food and Chemical Toxicology. 2011;49:1033-42.
261. Tobe H, Kubota M, Yamaguchi M, Kocha T, Aoyagi T. Apoptosis to HL-60 by Humulone. Biosci Biotechnol Biochem. 1997;61(6):1027-9.
262. Tyrrell E, Archer R, Skinner GA, Singh K, Colston K, Driver C. Structure elucidation and an investigation into the in vitro effects of hop acids on human cancer cells. Phytochemistry Letters. 2010;3:17-23.
263. Williamson EM. Synergy and other interactions in phytomedicines. Phytomedicine. 2001;8(5):401-9.
264. Arranz S, Chiva-Blanch G, Valderas-Martínez P, Medina-Remón A, Lamuela-Raventós RM, Estruch R. Wine, beer, alcohol and polyphenols on cardiovascular disease and cancer. Nutrients. 2012;4:759-81.
265. de Gaetano G, Costanzo S, Di Castelnuovo A, Badimon L, Bejko D, Alkerwi A, et al. Effects of moderate beer consumption on health and disease: A consensus document. Nutrition, Metabolism and Cardiovascular Diseases. 2016;26(6):443-67.
266. Gerhäuser C. Beer constituents as potential cancer chemopreventive agents. European Journal of Cancer. 2005;41:1941-54.