Valorisation of hop co-products for their phenolic content and antioxidant properties.

Thesis submitted to the University of Nottingham for the degree of

Doctor of Philosophy

November, 2024

Duncan Calvert

20405807

Supervisors: David Cook, David Gray, Arthur Gadon, Jacques Gros.

International Centre for Brewing Science, School of Biosciences

University of Nottingham

Acknowledgements

Firstly, I would like to thank my supervisors, in particular David Cook for his continued support in the research but also my development generally. When experiments were not going well David was able to help me see the positives and larger perspectives. David also challenged me and suggested me for things like conference talks, and although initially these were a little daunting, they were also insanely rewarding. David also put up with some of my very dense and descriptive drafts which he termed 'Duncan shorthand'. Having read through some of these drafts recently, I can only sympathise with him. David Gray has also provided me perspective and guidance for which I am thankful.

I would also like to thank my supervisors at AB Inbev. Jacques Gros and Arthur Gadon are passionate, cool people who helped me try and get the most out of hop tannins. They of course also provided the funding for my project, which allowed this keen hop nerd to keep looking at hops for a few more years.

When I first arrived at the University of Nottingham, it was the group of researchers in brewing sciences who helped me feel at home, gave me advise on experiments and drank ridiculously high adjunct and hoppy beers with me. These nice humans were Andrew Foulkes, Rodd White, Carolina Maia, Joshua Reid and Alex Jevons. I will always remember the initial period here as fun and exciting in part thanks to you guys. As time went on other fantastic people joined the department and helped make it feel like a fun cohesive group. These people were Joanna Yorke, Nigel Muchiwanga, Christabel Smith, Yishen Li, Giulia Roselli, Todd Isbell and Aline Brito. I look forward to catching up with all of you in the future. I would also like to thank Sue Clegg for having patience with me in the lab. Although I will never forget her look of rage when I used the viscometer without training!

Other mentions go to Tristan Dew for his insane knowledge of polyphenols and LC-MS which were so important to this project, and Pallab Borah for our stuff on hop leaf trichomes.

A final and important mention goes to K-RUT and whoever came up with idea of heat maps ordered by cluster analysis.

Table of Contents

List of abbreviations

Aqueous ethanolic solution 50% v/v	E50
Total phenol content	TPC
Total flavonol content	TFC
Proanthocyanidin content	PAC
Caffeic acid equivalent	CAE
Gallic acid equivalent	GAE
Procyanidin B3 equivalent	PB3E
Dry Material	DM
Calypso	CAL
Cascade	CAS
Contessa	CON
Young leaves	Y
Old leaves	O
Flower stage of development	FL
Middle stage of development	MID
Harvest stage of development	HV
Principal component analysis	PCA
Agglomerative hierarchical cluster	AHC
International calibration extract-3	ICE-3
Coumaroylquinic acid	COQA
Feruloylquinic acid	FQA

Caffeoylquinic acid	CQA
Dicaffeoylquinic acid	DCQA
Procyanidin B-type dimer	PB D
Quercetin 3-O-rutinoside	Q-RUT
Quercetin 3-O-glucoside	Q-GLUC
Quercetin 3-O-malonyl 6''-glucoside	Q-MG
Quercetin 3-O-neohesperidoside	Q-NEO
Quercetin tri glycoside	Q-TG
Quercetin	Q
Kaempferol	K
Kaempferol 3-O-rutinoside	K-RUT
Kaempferol 3-O-glucoside	K-GLUC
Kaempferol 3-O-galactoside	K-GALAC
Kaempferol 3-O-malonyl glucoside	K-MG
Kaempferol 3-O-neohesperidoside	K-NEO
Kaempferol 3-rhamnoside-7,4'-digalactoside	K-3-R-7,4-DIGALAC
Kaempferol 3,7'-diglucoside	K-3,7-DIGLUC
Washington State University	WSU
Extracted ion chromatogram	EIC
Total ion chromatogram	TIC
MS2	MS/MS
REF	Reference

Abstract

There is growing interest in the valorisation of agricultural and food processing residues for their functional bioactive compounds, due to high availability, low cost and alignment with a circular economy model. The harvesting and processing of hop cones for the brewing industry generates co-products which contain phenolic compounds of potential value to the cosmetic, food and beverage sectors due to their antioxidant properties. This study aimed to evaluate these co-products as a source of natural phenolics by producing extracts with high antioxidant activity using scalable, green extraction and purification techniques. Purified hop phenolic fractions were benchmarked against commercially available phenolic antioxidants produced from non-hop sources.

In experiments reported in Chapter 3 the focus was on optimising the extraction of phenolics from hop processing residue (cv. Herkules) using aqueous ethanolic solutions. A response surface methodology was adopted to evaluate the effects of; ethanol % of extraction solvent (30-90%), and solid: liquid ratio (100-150 mg/ml). Ethanol % was highly significant ($P<0.0001$) for the extraction of total phenolic content (TPC), proanthocyanidin content (PAC), xanthohumol, total alpha acids and Ferric Reducing Antioxidant Power (FRAP), whilst solid: liquid ratio did not significantly ($P>0.05$) impact on any of those response variables. A range of commercial enzymes (cellulases, pectinases and proteases) and incubation conditions were evaluated to see if they could increase the release of bound phenolics, however no treatments resulted in significantly greater extraction ($p>0.05$). It was concluded that optimal extraction conditions for the simultaneous extraction of TPC, PAC and xanthohumol were 50% ethanol (v/v) at 100mg/ml with no hydrolytic enzymes.

These extraction conditions were then applied to a range of hop co-products and non-hop materials (Chapter 4) to characterise their phenolic composition and antioxidant activity. Hop co-product extracts generally had higher phenolics content and antioxidant activities compared to non-hop materials. Phenolic structural elucidation and quantitation using LC-ESI-qTOF-MS/MS showed that prevalent hop co-product phenolic fractions include flavanols, B-type procyanidins, flavonol glycosides, prenylflavonols and chlorogenic acids. The phenolic profile of co-products depended on the co-product stream and hop variety, with prenylflavonols being abundant in CO_2 extract residues, catechins and procyanidins in T45 pelleting residues, and flavonol glycosides and chlorogenic acids in hop leaves. Notably,

Herkules CO_2 extract residue was found to be a rich source of prenylflavonoids, in particular xanthohumol (9.1 mg/g DM), a prenylflavonoid with potent hydroxyl and peroxyl scavenging activities and anti-cancer properties. Herkules CO_2 extract residue is also the most available material to industry and was therefore selected for subsequent attempts to generate purified, more concentrated hop phenolic extracts.

In Chapter 5 the use of different adsorption resins (PVPP and PAD950) and ultrafiltration membranes (UFX10, FS40, GR51) were investigated for the purification of phenolics from Herkules CO_2 extract residue. The phenolic purity and antioxidant activity of purified extracts from CO_2R-HERK strongly depended on the purification technique used. The highest purity extract was produced via adsorption with PAD950 resin, and was mainly composed of catechins, B-type procyanidins and flavonol 3-O-glycosides. Ultrafiltration was much less effective across a range of membrane cut-off sizes evaluated (10-100 kDa) indicating that PAD950 SPE offers greater phenolic specificity as compared to molecular weight size exclusion techniques. Langmuir isotherm modelling demonstrated high adsorption capacities of PVPP for hop phenolics (in particular xanthohumol), for both E0 and E50 feed solutions. However, recovery rates using ethanol, ethyl acetate and ammoniacal ethanol of varying strengths were generally ineffective for phenolic recovery using a fixed bed column setup.

Later work (Chapter 6, 7) comprehensively evaluated hop leaves as a source of phenolic antioxidants by sourcing three commercially significant varieties grown in Yakima, sampled at different developmental stages and crop years. The phenolic profile of hop leaves exhibited considerable structural diversity and differed significantly from that of respective cones. Kaempferol and quercetin 3-O-glycosides as well as chlorogenic acids were the most abundant sub-groups, with phenolic acids, procyanidins, prenylflavonoids and bitter resins also present. Multi-variate analysis indicated that the phenolic profile of hop leaves was primarily variety-dependent and driven largely by the composition of flavonol glycosides, with crop year and developmental stage showing smaller and less consistent effects. Compared to South African hop leaf extracts, Yakima hop leaf extracts exhibited significantly lower total phenolic contents but higher flavonol glycoside levels, highlighting the variability in phenolic content among hop leaf sources.

In Chapter 8 the antioxidant activities of hop leaf extracts were evaluated and correlated with phenolic compound concentrations in the samples. Pearson's correlation analysis revealed significant positive correlations for B-type procyanidins and catechins ($P<0.05$), whilst kaempferol 3-O-glycosides generally had negative correlations. Antioxidant analysis of pure chemical standards generally confirmed these findings and highlighted that leaf flavonol glycosides had significantly lower antioxidant activity compared to their respective aglycones, with kaempferol 3-O-glycosides exhibiting negligible DPPH and FRAP activity. Since

kaempferol 3-O-glycosides were generally the most abundant phenolics in hop leaf extracts, enzyme assisted hydrolysis was evaluated to improve the antioxidant properties of hop leaf extracts. Snailase treatment of leaf extracts achieved high hydrolysis rates (>99%) and significant increases in antioxidant activity depending on glycoside composition and antioxidant mechanism assayed. Cascade leaf extract exhibited the greatest increases in antioxidant activity (FRAP 4.5*) likely due to the high kaempferol 3-O-rutinoside content (400.56 mg/g). These findings highlight the potential for hydrolytic treatment of hop leaf extracts to generate natural antioxidants.

Chapter 1-Introduction

1.1-Background

A circular economy aims to redefine traditional production and consumption systems using closed-loop cycles that minimise waste and upcycle waste materials into products of value to different sectors. The ability to repurpose waste aligns with increasing sustainability demands and the drive to reduce CO_2 emissions. The agricultural, food and beverage industries generate large volumes of processing waste, termed co-products, which includes the peels, skins, pomaces, and leaves of commonly processed fruits and vegetables (Mir-Cerdà et al., 2023). These residues have previously been restricted for use as animal feed or fertiliser, however recently more valuable applications are emerging primarily due to their rich phytochemical content, particularly phenolic compounds (Rațu et al., 2023).

Recently phenolics have gained attention for their functional properties such as anti-oxidant, anti-microbial, and anti-carcinogenic which could provide value to diverse industries such as the nutraceutical (Shahidi et al., 2019), pharmaceutical (Osorio et al., 2021), food (Rațu et al., 2023) and beverage (Routray and Orsat, 2019) sectors. As a result, attention in the last decade has focused on the recovery of phenolic compounds from co-products using sustainable non-toxic technologies that align with safety regulations. A recent report titled 'Polyphenols Market Analysis' predicted an 8.72% compound annual growth rate from 2023 to 2028 which is attributed to increasingly health-conscious consumers and a growing demand for functional foods and nutraceuticals (Mordor Intelligence, n.d.). Along with their generally recognised as safe status, this is expected to further broaden the scope for applications of phenolics in different sectors.

The harvesting, processing, and use of hop cones in the brewing process generates 'co-products' including leaves, shoots, processing residues and brewing residues. These are

produced in substantial quantities, but are mostly used as fertiliser due to their high nitrogen content (up to 40 g/kg in hop leaves) (Afonso et al., 2023). However, research has highlighted that hop co-products are a rich source of bioactive phenolic compounds and have therefore been evaluated for a large range of functional properties including, vascular-protective (Luzak et al., 2016), estrogenic (Chadwick et al., 2004), antimicrobial (Bartmańska et al., 2018) and anti-inflammatory (Caban et al., 2020). Most of these functional properties of hop phenolics are generally attributable to their ability to act as potent antioxidants through different mechanisms such as scavenging reactive oxygen species (ROS) and metal chelation. Thus, there is promise in the use of hop co-products as a source of natural phenolics for the development of high antioxidant extracts which could provide value to diverse industries.

1.2-Literature Review

1.2.1-Hops

1.2.1.1-General

Hop cones are the inflorescences of the species *Humulus Lupulus* L., and are one of the four main ingridients of beer along with malt, yeast and water. *Humulus Lupulus* belongs to the Cannabaceae family, which also includes hemp, and is classified within the Urticales order. Figure 1.1 outlines the structure of hop cones which consist of a strig, bracts and lupulin glands. Hops play an important role in imparting the characteristic bitterness and aroma to beer which stems from resin and oil compounds respectively. These compounds and their use in brewing represent the main source of economic value derived from the hop plant, which drives its cultivation around the world with 62,802 ha (2022) devoted to hops worldwide (Barth-Haas-Group, 2023). Hops are predominantly cultivated in Germany and the USA which together account for around 60% of the hop area under cultivation but they are also grown in other countries around the world such as the UK, Slovenia, New Zealand and

Australia (Almaguer et al., 2014).

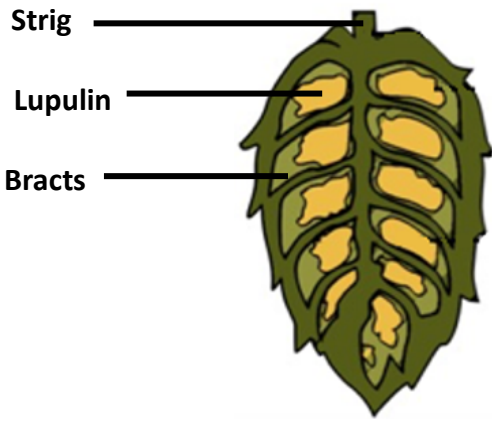


Figure 1.1: The structure of hop cones (Almaguer et al., 2014).

1.2.1.2-Hop composition and their use in beer

Hop cones contains various primary metabolites which are essential for growth including carbohydrates, sugars, lipids, amino acids and proteins (Schönberger and Kostelecky, 2011). The chemical composition of a typical fresh hop cone at 10% moisture is outlined in Table 1.1. Proteins, carbohydrates, and polyphenols are predominantly located in the bracts of the hop cone, whilst bitter resins and aromatic oils are located in the lupulin glands and accumulate over the course of cone maturation (Kavalier et al., 2011).

Table 1.1: General chemical composition of dry hop cones (Almaguer et al., 2014).

Constituent	Amount (%)
Total resins	15–30
Essential oil	0.2–3.5
Proteins	15
Monosaccharides	2
Polyphenols (tannins)	4-10
Pectins	2
Amino acids	0.1
Waxes and steroids	traces–25
Ash	8
Moisture	10
Cellulose	43

Hops are a particularly rich source of secondary metabolites which are broadly classified into resins, oils and phenolics, a breakdown of which is presented in Figure 1.1. Bitter resins and aromatic essential oils are essential for beer production contributing to bitterness, flavour and aroma, as well as enhancing foam retention and microbial stability (Schönberger and Kostelecky, 2011).

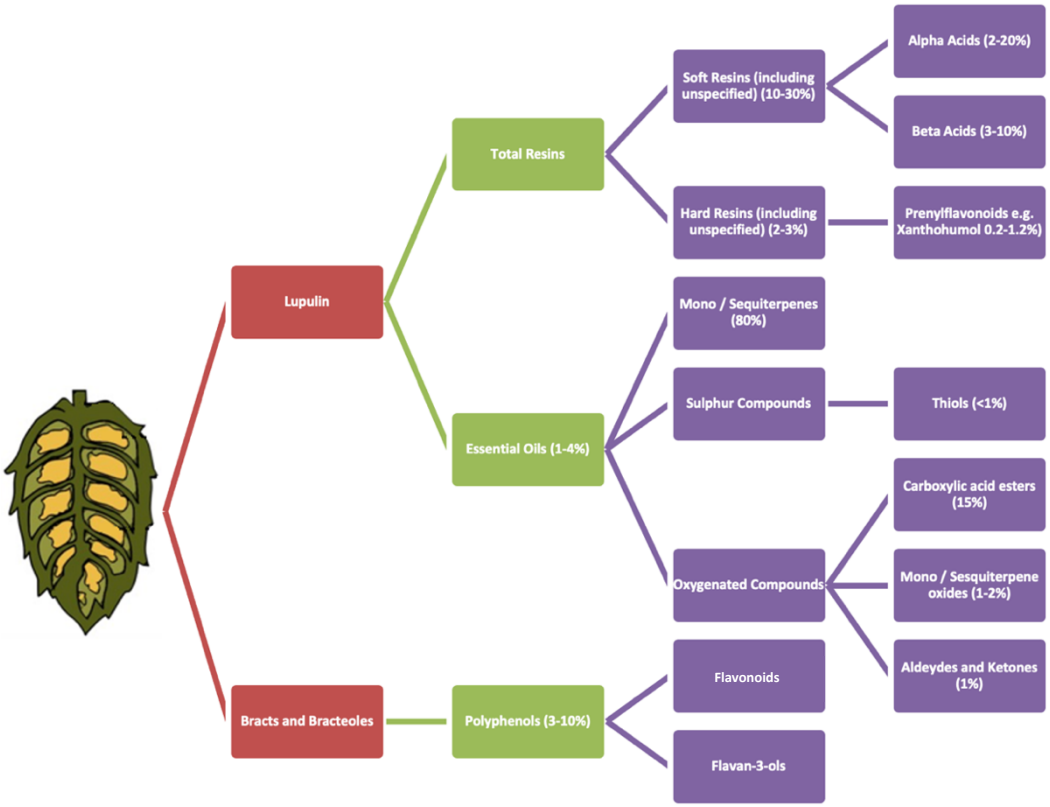


Figure 1.1: Classification of hop secondary metabolites (Biendl et al., 2015).

Hops have a particularly high bitter resin content, which can reach up to 30% in hop cones. Resins, the most abundant secondary metabolites in hops, are divided into hard and soft resins based on their solubility, with hard resins soluble in methanol but not hexane, whilst soft resins are soluble in hexane. The soft resin fraction predominantly consists of humulones (α-acids) and lupulones (β-acids) which are di- and tri-prenylated derivatives of phloroglucinol. These compounds consist of homologs which can be identified by their alkanoyl side chain such as humulone, co-humulone and ad-humulone (α-acids) with other structural variations ad-pre-, pre- and post-homologs present at lower levels (Almaguer et al., 2014). During wort boiling α-acids isomerise into cis- and trans-stereoisomers which increases their solubility and bitterness intensity (Ting and Ryder, 2017). Although lupulones exhibit poor solubility in beer, their oxidative degradation products such as hydroxytricyclolupulones, hulupones and hulupinic acid, can contribute to bitterness via additive and synergetic effects (Algazzali and Shellhammer, 2016).

The essential oil fraction contributes to the aroma of beer and comprises over 440 volatile compounds. Dried hop cones typically contain 0.2-3.5% essential oil which is predominantly comprised of hydrocarbons such as monoterpenes (e.g. myrcene, limonene), sesquiterpenes (e.g. caryophyllene, humulene) and their oxygenated derivatives, as seen in Figure 1.1 (Schönberger and Kostelecky, 2011). The influence of these compounds on the aroma characteristics of beer depends largely on their volatility, sensory thresholds and point of addition in the brewing process.

Given the importance of phenolics on the antioxidant properties of hop co-products, plant phenolic and their occurrence in hop cones are discussed in more detail in the following section.

1.2.2-Plant phenolics

Plant phenolics are a structurally diverse group of secondary metabolites that share a common structure of an aromatic ring with at least one hydroxyl groups. They are widely

distributed across different plant species and contribute to many important properties in foods and beverages such as astringency and colour (Adebooye et al., 2018). Phenolics are synthesized in plants through the shikimate and phenylpropanoid pathways partly as a defence response to abiotic and biotic stresses e.g. UV radiation and pathogens which can promote the accumulation of these compounds throughout the hop plant (Kunej et al., 2020).

1.2.2.1-Hop Phenolics

Plants typically have higher phenolic contents in leaves possibly as a defence against UV radiation, however in hops they are predominantly found in the surrounding bracts of hop cones (Kurumatani et al., 2005), with their presence in lower concentrations throughout other parts of the plant such as leaves, shoots and seeds (Abram et al., 2015, Alonso-Esteban et al., 2019).

Hop polyphenols comprise approximately 4-10% of the total weight of hop cones (at 10% moisture) (Biendl et al., 2015). These compounds are structurally diverse and over 100 different compounds have been identified in hops which can be classified by their structure into four main groups; flavonols, flavan-3-ols, phenolic carboxylic acids and 'others' as seen in Figure 1.2 (Schönberger and Kostelecky, 2011).

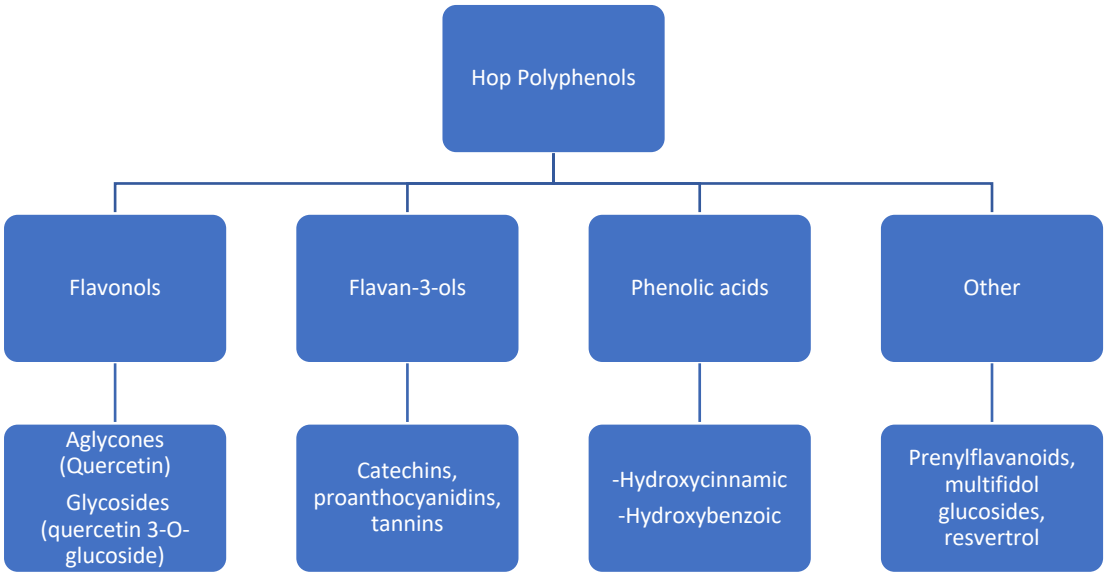


Figure 1.2: The different groups of hop polyphenols and their constituents (Biendl et al., 2015).

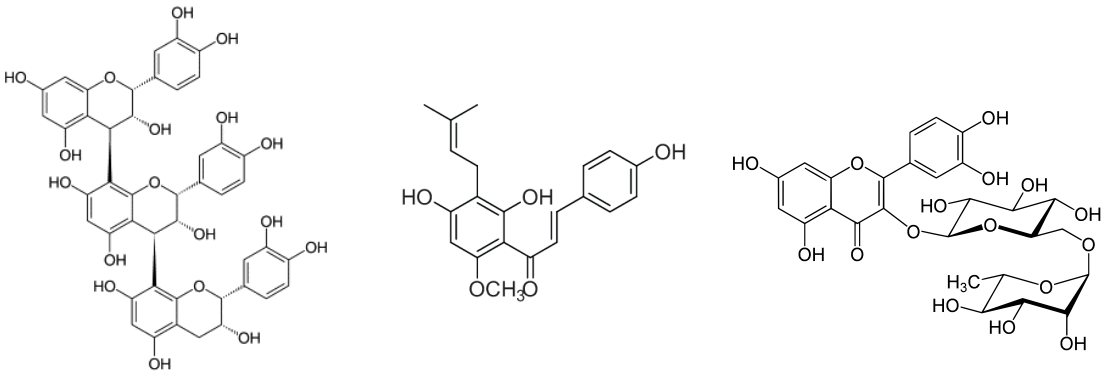
1.2.2.1.1-Flavanoids and proanthocyanidins

The most abundant phenolics in hop cones are B-type proanthocyanidins and prenylflavonoids with multifidol glucosides, flavonol glycosides and chlorogenic acids present at lower concentrations depending on variety and origin (Kavalier et al., 2011). Flavonols and flavan-3-ols are classified as flavonoids as they are derived from flavone. Flavonols are distinguished by a ketone group at the C4 position on the C-ring and in hops are predominantly glycosidically bound to mono-, di- and tri-glycosides (Dušek et al., 2021). Flavan-3-ols such as catechin and epicatechin occur in free form but are also monomers for higher polymeric structures such as oligomeric proanthocyanidins (up to 8 units) and tannins (20 units or more) (Keskin et al., 2019).

Proanthocyanidins are prevalent across different plant species and can range from 0.5-5% in hop cones depending on cultivar and the method used for analysis (Stevens et al., 2002). Hops are predominantly composed of B-type proanthocyanidins which are present at much higher levels that A-type proanthocyanidins. Procyanidins, composed of catechin and epicatechin are present at the highest levels in hops, however prodelphinidins composed of gallocatechins are also present at low concentrations (Li and Deinzer, 2009). These condensed tannins derive their name from the formation of anthocyanidins upon acid depolymerization (Li and Deinzer, 2006). Proanthocyanidins have received much attention for their health and antioxidant properties which are thought to be greater than that of their respective hop flavanols which when oxidised can form prooxidant quinones (Bors et al., 2000). Antioxidant activity may be related to chain length, for example higher molecular weight proanthocyanidins seem to be more effective against liposome oxidation (Lotito et al., 2000).

Prenylflavonoids are a sub-group of flavonoids and are characterised by the presence of one or more prenyl functional groups which increase their hydrophobicity. Xanthohumol is the

principle prenylflavonoid of hop cones comprising up to 1.7% in dried cones (Stevens et al., 1997). Other prenylflavonoids such as 8-prenylnaringenin (8-PN), 6-prenylnaringenin (6-PN), isoxanthohumol and desmethylxanthohumol are present at lower levels. Hop estrogenic activity is mainly attributed to 8-PN, which is considered to be one of the most potent phytoestrogens (Possemiers et al., 2006). New structurally related prenylflavonoids often referred to as xanthohumols continue to be identified in hops (Stevens et al., 2000, Forino et al., 2016). Generally polyphenols are concentrated in the bract of the hop cone, however the prenylflavonoids are disproportionally found in the lupulin glands which contain the enzymes responsible for catalysing prenylation (Roberts and Wilson, 2006). The chemical structures of the most abundant flavonoid groups found in hops are presented in Figure 1.3.



Proanthocyanidins | **Prenylflavanols** | **Flavonol glycosides**

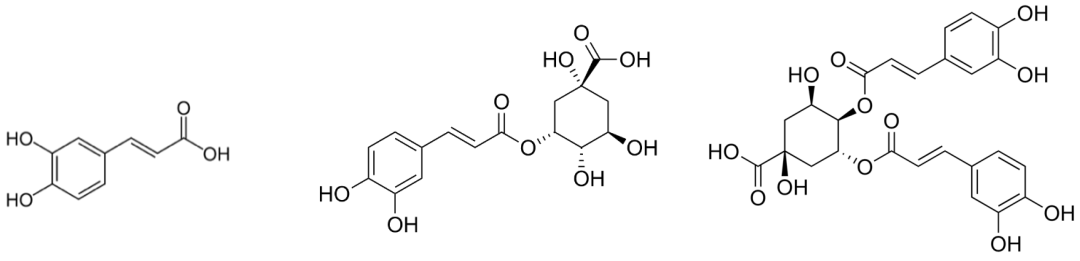
Procyanidin B3 | Xanthohumol | Quercetin 3-O-rutinsodie

Figure 1.3: Chemical structures of the abundant flavonoids found in hop cones.

1.2.2.1.2-Chlorogenic acids

Chlorogenic acids are formed between quinic acid and different hydroxycinnamic acids, the most common being caffeic, ferulic and p-coumaric. This produces compounds such as caffeoylquinic, dicaffeoylquinic, feruloylquinic and coumaroylquinic acid, depending on the type and number of phenolic acid units (Lu et al., 2020). These are found in a variety of plant

species and foods however are most prominent in coffee where they contribute to astringent and bitter properties (dos Santos Scholz et al., 2018). Chlorogenic acids, in particular caffeoylquinic acids have been identified in hop cones and are generally present at higher levels than phenolic acids, but at lower levels compared to B-type proanthocyanidns and prenylflavonoids (Schmidt and Biendl, 2023b). Activity guided studies have associated chlorogenic acids in hops with anti-inflammatory (Nicacio et al., 2022) and Alzheimer's combating properties (Palmioli et al., 2022). Figure 1.4 highlights the different chemical structures of caffeic, 3-chlorogenic and 4,5-dicaffeoylquinic acid.



Caffeic acid **3-Chlorogenic acid** **4,5-Dicaffeoylquinic acid**

Figure 1.4: Chemical structure of caffeic, mono- and di-caffeoylquinic acid.

1.2.2.1.3-Multifidols

Multifidols are phloroglucinol derivatives with anti-inflammatory properties first identified in hop cones by Bohr et al. (2005). As is the case with flavonols, these are predominantly present in glycosidically bound forms and have been reported in hop cones at concentrations up to 311.4 mg/100g depending on the variety and cultivation location (Schmidt and Biendl, 2023b).

Table 1.2 presents a summary of literature relating to the impact of different factors on hop phenolic composition.

Table 1.2: Literature summary on the impact of botanical origin, geographical origin, growing techniques, harvest maturity and processing on the phenolic composition of hop cones.

Factor	Hop cones analysed	Conclusions	Reference
Botanical Origin	Czech hop cones (7 varieties)	-Saaz had the highest polyphenol content (in particular catechin). -Strong varietal impact observed.	(Jelinek et al., 2010)
	Hop cones (13 varieties)	-Minor differences in proanthocyanidin composition observed between varieties.	(Li and Deinzer, 2006)
	Hop cones (9 varieties)	Variety significantly impacted stilbene content, including resveratrol and piceid isomers.	(Jerkovic et al., 2005)
	Czech Hop cones (5 varieties)	Flavonol glycosides profile can be used to identify varieties across multiple crop years.	(Dušek et al., 2021)
	Polyphenols / Czech varieties	Proanthocyanidin composition depended on geographical origin.	(Olšovská et al., 2013)
Geographical Factors	German and American hop cones (11 varieties)	Low molecular weight phenolic content differed according to growing location.	(Forster et al., 2002)
Harvest Maturity	Hop cones	-Earlier harvested samples exhibited increased anti-adipocyte differentiation activity.	(Inui et al., 2017)
	English hop cones (5 varieties)	Prenylflavonoids and bitter resins increased at different rates from the start of cone development.	(De Keukeleire et al., 2003)
	Hop cones	-Proanthocyanidins decreased over maturity however this was variety dependent.	(Kavalier et al., 2011)
Processing/ Storage	T90 pellets	Polyphenols decreased 30-40% after 12 months with Saaz the most stable.	(Mikyška and Krofta, 2012)
	Hop cones	High-pressure processing (HPP) increased antiradical activity and phenolic content.	(Mikyška et al., 2015)
	Hop cones	Hop kilning slightly reduced DPPH radical scavenging activity (approximately 5%).	(Krofta et al., 2008)

1.2.3-Hop Co-products

Hop co-products can be categorised into three main streams based on their origin: cone harvesting, processing, or brewing (Figure 1.5). During harvesting, cones are separated from leaves and stems resulting in agricultural co-products located at the farm. Cones are then kilned, milled, and often processed into bittering and aroma extracts for brewing, with the resulting vegetative material termed processing residues. When cones or pellets are used

directly in the brewing process such as in kettle and dry hopping, the non-extractable spent material is termed brewing residue. Therefore, processing and brewing residues primarily consist of cone derived materials, whilst the harvesting and separation of hop cones produces co-products made up of hop leaf, stem and shoot biomass (Figure 1.5) which contain different biologically active molecules (Astray et al., 2020, Jacquin et al., 2022, Maietti et al., 2017).

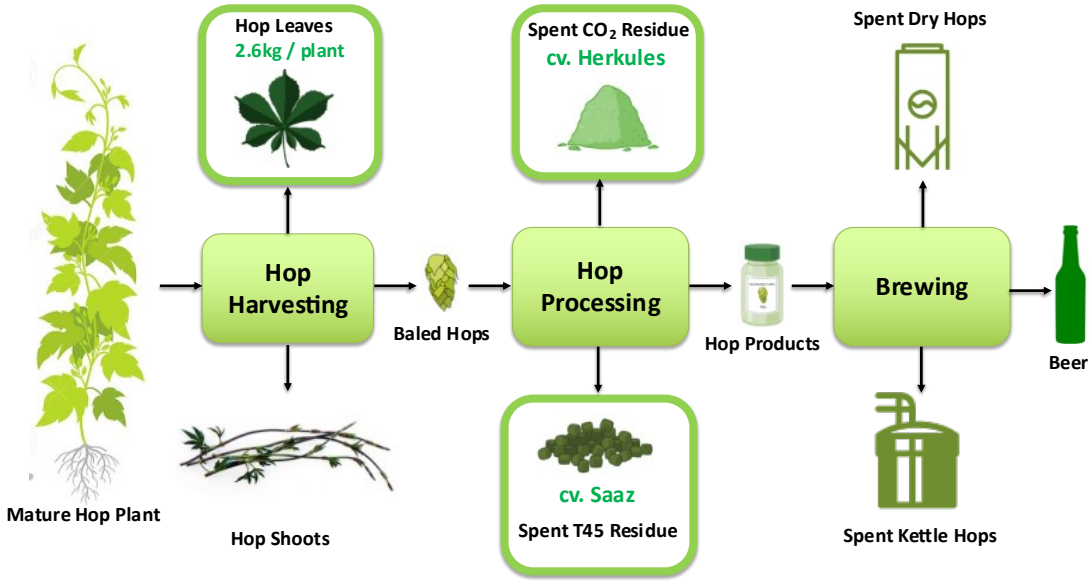


Figure 1.5: Hop co-product streams from the harvesting, processing, and brewing of hops.

1.2.3.1-Hop Processing Co-Products

Extraction and separation of hop bittering and aroma components from hop cones into extracts provides improved consistency, stability, utilisation and handling for brewers (Knez Hrnčič et al., 2019). Only approximately 20% of a hop cone's constituents have brewing value, and for this reason much is processed into lupulin enriched pellets or extracts. The most common methods for hop processing are CO_2 extraction (chemical extraction) and T45 pelleting (mechanical separation), the steps of which are outlined in Figure 1.6. The composition and properties of the residue material are mainly determined by processing

method and conditions employed as well as the variety and location of cultivation of the initial hop material (Bartmańska et al., 2018).

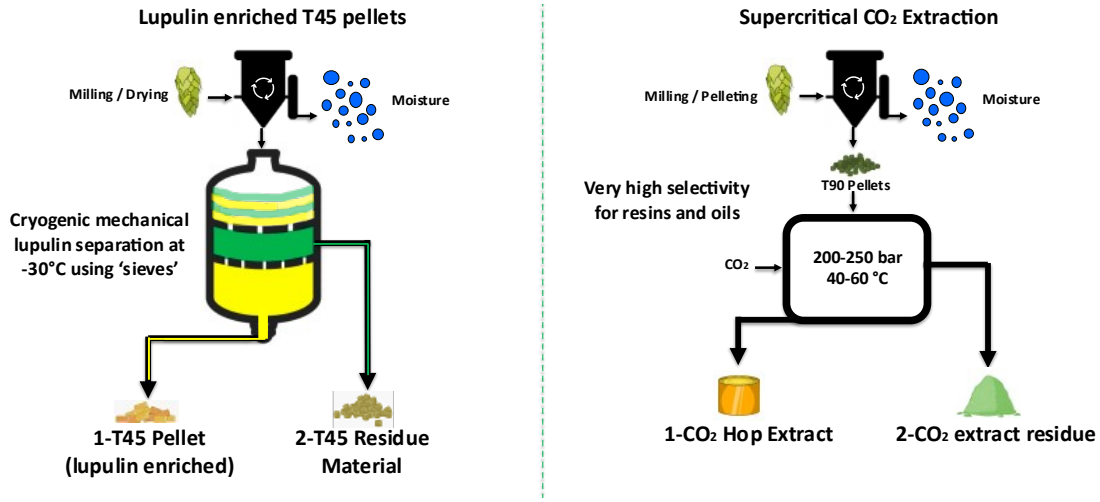


Figure 1.6: Outline of the processing steps for T45 pelleting and CO_2 extraction of hops.

Supercritical CO_2 is a highly apolar solvent and therefore has high selectivity for the bitter acids that are extracted for brewing usage. It is however a poor solvent for phenolics and thus CO_2R materials in theory contain higher concentrations of polyphenols (including prenylflavonoids and proanthocyanidins) compared to the respective cones, assuming no phenolic degradation takes place during processing (Jaskula-Goiris et al., 2014). Prenylflavonoids such as xanthohumol can be extracted with CO_2 extraction, but only at much higher pressures (>1000 bar) than those used for conventional resin and oil extraction (200-250 bar) (Schmidt et al., 2005).

For T45 pellet (T45P) production cones are cooled to -30 °C, crushed with a hammer mill (2-8 mm), homogenised, and 'sieved' to separate the lupulin glands from bract and other vegetative material. Bitter resins, aroma compounds and prenylflavonoids are predominantly found in the lupulin glands and are therefore found in higher concentrations in the T45P (Roberts, 2016). Whereas other polyphenols predominantly located in the bracts should

theoretically be concentrated in the residue (T45R) (Biendl et al., 2015, Schönberger, 2006). The two main types of pellets are T90 (non-enriched) and T45 (lupulin enriched) however there is trend towards further enrichment e.g., cryo-hops where practically only the lupulin glands remain (Hughes and Simpson, 1993). The current literature on the phenolic composition of hop processing residues and their evaluated application is summarised in Table 1.3.

Table 1.3: Literature reports on phenolic content of hop processing co-product extracts.

Material	Variety	Evaluated Application	Phenolic content (mg/g DM)	Source	Reference
CO_2R	Saaz	Improving flavour quality and stability of pilsner beers.	TPC-218 Procyanidin B3-9.0 Catechin-17.5 Coumaric acid-0.40 Ferulic acid-0.20 Quercetin 3-O-rutinoside -4.6 Kaempferol-3-O-glucoside-2.6 8-PN-0.40 Xanthohumol-7.3	Joh. Barth & Sohn (Nürn-berg, Germany).	(Jaskula-Goiris et al., 2014).
CO_2R	Vital	Alleviation of symptoms of menopausal disorders.	-Desmethylxathohumol-0.97 -8-PN-0.17 -6-PN-0.47 -Xanthohumol-9.20 -Isoxanthohumol-0.29 Alpha acids-10.04 Beta acids-n.d	(50 °C, 290 bar). prepared by Flaveko Trade, Czech Republic.	(Karabín et al., 2021).
CO_2R	Magnum	Flavouring potential of hop polyphenols in beer.	TPC-18.8 Xanthohumol-5.33 Quercetin-3-O-glucoside-0.66 Coumaric acid-0.04 Ferulic acid-0.062 Catechin-1.06 Procyanidin B3-0.47 Prodelphinidin B3-0.08	Botanix Ltd. (Paddock Wood, England).	(Goiris et al., 2014).
CO_2R	-	Blood platelet aggregation and anticoagulant activity.	Chlorogenic acids-76.7 Catechin-27.4 Epicatechin-40.8 PAC-233.1 Flavonol glycosides-244.4	Fertilizer Research Institute Pulawy (Pulawy, Poland).	(Luzak et al., 2016).
CO_2R	-	Antiproliferative effect on human umbilical vein endothelial cells.	TPC-239.4 TFC-117.6 PAC-93.1	Fertilizer Research Institute Puławy (Poland).	(Boncler et al., 2014).
CO_2R / T45R	Vital / Saaz	Improving haze and flavour stability of beer.	**Vital CO_2R-TPC-50.0** Catechin-1.41 Epicatecin-0.81 Ferulic acid-0.02 **Saaz T45R-TPC-95.0** Catechin-3.31 Epicatechin-52.3 Ferulic acid-0.03	Bohemia Hop Co. Ltd (Czech Republic).	(Jelínek et al., 2014).
T45R	Saaz	Acceleration of lautering.	TPC-87.0 Alpha acids-5.0 Beta acids-15.0 Total oil-0.9 **Resulting hop extract:** Catechin-3027 mg/L Epicatechin-405 mg/L Quercetin-4.6 mg/L Quercetin 3-O-glucoside-191 mg/L Quercetin 3-O-rutinoside-53 mg/L	HMELARSTVI, cooperative Žatec (Czech Republic).	(Karabín et al., 2018).

CO_2R-Hop residue after CO_2 extraction. **T45R**-Hop residue after T45 pelleting.
TPC-Total phenolic content. **TFC**-Total flavonol content. **PAC**-Proanthocyanidin content.

Saaz is a landrace aroma variety with notoriously low and variable α-acid contents and as a result is often processed into T45P's to concentrate and standardise resin and oil contents (Nesvadba et al., 2021). Saaz has also consistently been shown to have the highest level of phenolic compounds, in particular B-type proanthocyanidins, compared to other varieties (Gadon et al., 2019a). Herkules is currently the leading European bittering variety due to its

high α-acid production, good yields and favourable agronomic characteristics, and is therefore the variety of choice for CO_2 extraction (Forster and Gahr, 2019). Considering this, Saaz T45R and Herkules CO_2R are an abundant, potentially rich sources of phenolic compounds with potential for valorisation. CO_2R and T45R have been evaluated in the literature as a source of phenolics, however, there is a lack of quantitative data on non-prenylated hop polyphenols (flavonol glycosides, procyanidins) which is important in assessing the feasibility of their valorisation as a source of phenolic antioxidants. It is also unclear whether these compounds degrade during the processing of hop cones, or if they remain intact potentially leading to higher concentrations in processing residues.

1.2.3.2-Hop Agricultural Co-products

Leaves are generally the primary components of plant agri-food by-products and contain phenolics including flavonol glycosides, chlorogenic acids and procyanidins (Andrade et al., 2022). Hop harvesting co-products are produced in significant quantities (~2.6 kg/plant) but are yet to be fully exploited (Abram et al., 2015). Leaves represent around 25% dry weight (DW) (Figure 1.7) of the plant with stems and cones representing somewhat higher proportions at 30.5% DW and 44.5% DW respectively depending on variety (Sarraf et al., 2012).

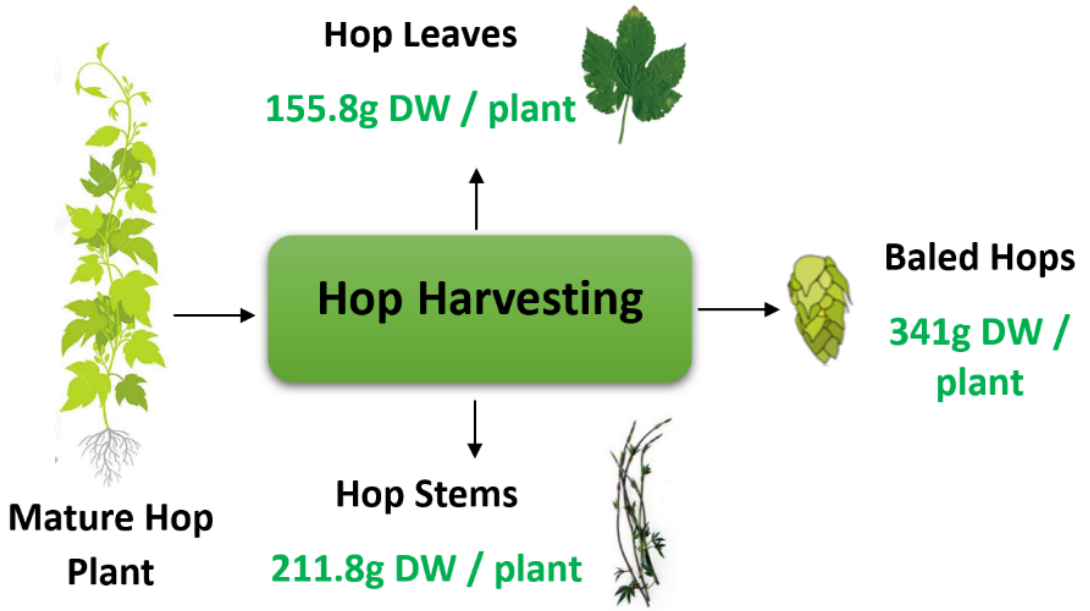


Figure 1.7: Hop materials generated from the harvesting of hop cones. Mass balance calculated from yield analysis by Sarraf and averaged over five varieties (Sarraf et al., 2012).

Various glandular trichomes have been found on the lower leaf epidermis (Mishra et al., 2020), which differ morphologically from cone trichomes and contain low levels of bitter acids, prenylflavonoids and terpenes (Nagel et al., 2008). The epicuticular wax layer of hop leaves also seem to contain esters, hydrocarbons aldehydes with primary alcohols making up the largest component at 54% (Gülz et al., 1993). The presence of bitter resins and prenylflavonoids has been confirmed on day of harvest in hop leaves of commercial cultivars but at very low concentrations (below 0.02%) (De Keukeleire et al., 2003).

Abram et al. (2015) reported that the hop leaf phenolic content is 3-30 fold lower than their respective cones, whereas Keskin et al. (2019) reported similar concentrations for leaves and cones (7.12mg GAE/g to 6.86mg GAE/g respectively). Flavonol glycosides, procyanidins and chlorogenic acids have been tentatively identified in the leaves of commercial cultivars using LC-MS/MS (Morcol et al., 2021). Similarly, glycosides of quercetin and kaempferol were reported as the most abundant phenolics in hop shoots (Maietti et al., 2017). A literature summary on the main factors impacting hop leaf phenolic composition is presented in Table 1.4. Table 1.4 also highlights the variation in extraction conditions employed in the different

studies thereby making comparisons challenging. The influence of extraction conditions on the phenolic composition of extracts is discussed in Chapter 3.

Table 1.4: Literature summary on factors impacting hop leaf phenolic composition and antioxidant activity.

Analysis	Extraction Conditions	Conclusions	Reference
		Botanical Origin	
TPC, DPPH (IC_{50}), TFC.	-Ethanol (50): Water (50). - 33.33 mg/30 ml, 40 °C in ultrasonic bath for 3 h.	-Significant hop leaf varietal differences. -Victoria-(23.6 mg GAE/g) > Spalt > Cascade.	(Iglesias et al., 2021).
TPC, TFC, DPPH, ABTS, Chlorophyll, TC (carotenoids).	-Ethanol (96): Water (4). - Ultrasound assisted (USA), 1 g powder/15 ml, 25 °C.	-Hop leaf variety, drying treatment as well as interaction were all significant to different degrees. -Oven dried leaves had lower TPC, but higher carotenoids compared to freeze dried leaves. -Chinook (39 mg GAE/g) > Columbus > Cascade.	(Macchioni et al., 2022).
TPC, PAC, Prenylflavonoids, α / β-acids.	-	-Significant hop leaf varietal differences. -Nugget leaves highest α-acids (0.9 mg/g DM), Brewers Gold highest xanthohumol (0.14 mg/g DM) and Galena highest PAC (11 mg/g DM) / TPC (31.8 mg/g DM). Nugget cones slightly higher TPC (46 mg/g DM) PA (17 mg/g DM) but substantially higher α-acids (108 mg/g DM).	(Sarraf et al., 2012).
Targeted / untargeted (UPLC-QTof-MS), gland counts.	-Methanol (80): Water (20) with sonication.	-*H. neomexicanus* leaf phytochemical composition significantly different to European commercial cultivars with higher α/β-acids, prenylflavonoids and flavonol glycosides (although lower phenolic acids). Presence of caffeoylquinic acids. - *H. neomexicanus* significantly higher glandular trichome densities. -No significant differences in flavanols and proanthocyanidins.	(Morcol et al., 2021).
HPLC-DAD.	-Methanol with 0.01% formic acid.	-Significant differences between English varieties for desmethylxanthohumol, xanthohumol, α/β-acid content of hop leaves on day of harvest. -Admiral and Target highest concentrations of xanthohumol, α/β-acids whilst still very low. Challenger, WGV and Golding very low for all compounds.	(De Keukeleire et al., 2003).
TPC, DPPH, TFC and HPLC-UV.	-Methanol (75): Water (25).	-Leaf TPC ranged from 31.4-63.59 mg rutin equivalent/g DM whilst cones ranged from 71.72-78.18 mg rutin equivalent/g DM depending on variety. -Leaf xanthohumol concentration ranged from 0.014-0.08 mg/g DM whilst cones ranged from 1.12-2.14 mg/g DM.	(Stanius et al., 2022).
TPC, DPPH, ABTS, FRAP and UHPLC-MS / MS.	-Ethanol (80): Water (20).	-Leaf TPC varied from 3.92-8.00 mg GAE/g DM depending on variety and extraction method. -Coumaroylquinic acids, flavonol glycosides, flavanols, prenylflavonoids and bitter acids identified in Cascade leaves.	(Chiancone et al., 2023).
		Harvest Maturity and Environmental Factors	
TPC, TFC, DPPH, BCLM (β-caroten	-Methanol. -50 mg/ml.	-Slovak hop leaves exhibited varietal differences for DPPH reduction which increased during vegetative period from May 5th to July 7th reaching highest activity at harvest (Sep 9th).	(Pšenáková et al., 2010).

linoleate model system).			
TPC, DPPH, FRAP.	-Methanol, ethanol, ethyl and isopropyl alcohol at three concentrations. -Extraction time at 15, 30 and 60 min. -Ultrasound-assisted extraction; 40kHz.	-Young leaves (upper) at the beginning of vegetation showed high antioxidant activity. -Antioxidant activity differed depending on plant material harvesting time possibly a result of climate conditions of different years.	(Muzykiewicz et al., 2019).
TPC, TFC.	-Methanol (95): Water (5). -Rotary Evaporator.	-Climate impacted leaf polyphenol composition. -Mainly depended on vegetal period with leaf TPC decreasing from end of June to September. Similar effect for TFC. -Crop year and variety also significant (Bor (14.34 mg GAE/g > Sladek > premiant). -Antibacterial activity more related to bacterial strain than hop variety.	(Urgeova and Polivka, 2009).
UPLC-QTof-MS.	-Methanol (80): Water (20). -Sonicated (30 min) -10 mg/ml.	-Environmental stress e.g., drought impacted dihydromyricetin in hop leaves which was not found in cones. Environmental stress less significant than cultivar. -Progressive to severity of drought with TPC generally higher in plants not watered.	(Morcol et al., 2020).
-Anatomical analysis (SEM).	-Methanol. -Stirred for 3 h.	-Quantities of elements in hop leaves influenced by the timing and plan of fertiliser treatment. -It also increased α/β-acids and total oil concentrations in cones.	(Rodolfi et al., 2021).
TPC, α/β-acids and prenylflavonoids.	-	-Leaf TPC increased from mid-July to beginning of Aug and then decreases for all cultivars and conditions. Consistent amongst cultivars and stress factors. -Xanthohumol content in leaves was variety specific (*Taurus* highest-0.09%) and decreased in July. -Highest content found in South African variety Southern Star (14.28 mg GAE/g DM). Lowest found in Slovenian varieties Celeia, Aurora, however this isn't reflected in cone polyphenol composition.	(Ceh et al., 2007).
UHPLC-MS, cytotoxicity and cell metabolism assays.	Methanol (80): Water (20).	-Young Cascade hop leaves (collected 6 weeks after potting) contained oxidised bitter acids and feruleoylquinic acids but not xanthohumol. -Hop leaf extract encapsulated in rapeseed lecithin nanoliposomes exhibited anti-inflammatory activity.	(Velot et al., 2022).

TPC: Total phenol content. **TFC:** Total flavonol content. **PAC**: Proanthocyanidin content. **GAE**: Gallic acid equivalent. **DM**: Dry material. **FRAP**-Ferric reducing antioxidant power.

1.2.3.3-A comparison of the characteristics of different hop co-products

Whilst spent hop from brewing has been well evaluated, it is only recently that efforts have focused on hop harvesting and processing co-products to assess their composition, *in vitro* activity and potential application (Sun et al., 2022). Hop co-products from these different streams (Figure 1.6) have inherent advantages and disadvantages for use as a source of natural phenolic for valorisation. Brewing co-products usually emerge from the kettle or fermenter as a crude slurry 'contaminated' with yeast (spent dry hops) or protein (spent kettle hops) and therefore require purification and stabilisation due to high water content (Bravi et al., 2021, Olivares-Galván et al., 2022). The high temperatures of wort boiling can also lead to phenolic degradation (spent kettle hops), and the more water-soluble phenolic compounds can also be lost through extraction into wort and beer, particularly during dry hopping due to longer contact times and ethanol content which can increase phenolic extraction (Alfeo et al., 2023, Cortese et al., 2020). However, spent hop from brewing has been evaluated for antioxidant activity (Censi et al., 2021), as an eco-friendly repellents for insect pests (Bedini et al., 2015) and as a source of prenylflavonoids (de Andrade Silva et al., 2023).

Hop leaves and stems generated from the harvesting and separation of cones also have a high moisture content (approx. 80%) and require stabilisation to prevent degradation and maintain bioactive properties. This could be performed using the cone kilning facilities located at the hop farm however hop kilning is the single most significant GWP input in hop production (28.19%) (Bristol, 2022). Macchioni et al. (2021) also demonstrated that kiln dried hop leaves exhibited lower antioxidant activity compared to the respective freeze-dried leaves. Hop processing co-products conversely have a low moisture content, small particle size and are likely present at a processing facility making them highly suited as a starting material for phenolic extraction (Mirowski et al., 2021).

The generation of these co-products, and therefore their availability for phenolic extraction varies for each stream. Spent kettle hop residue from brewing is being generated in lower quantities due to the trend towards lower kettle hopping rates (Aron and Shellhammer, 2010). However, spent dry hop residue is becoming more abundant due to higher dry hop additions stemming from the increasing popularity of high hop aroma in craft beer (Reid et al., 2020). Processing co-products are becoming more abundant as more brewers opt for CO_2 hop extracts and lupulin enriched pellets over whole cones and pellets (Sanz et al., 2019). Traditionally, T90 and T45 pellets have been the primary forms, with T45 pellets offering lupulin enrichment. However, recent trends, such as the development of cryo-hops, aim for even higher lupulin enrichment, which further increases the abundance of the resulting residue. The availability of hop leaves and stems depends on hop acreage but also variety which impacts the proportions of cones, leaves and stems produced (Sarraf et al., 2012).

1.2.4-Extraction of phenolics from plant materials

Solid-liquid extraction has been the standard technique for extracting phenolics from plant materials with different factors altered for optimal extraction, which impacts the content and composition of phenolics extracted. These include pH, duration, temperature, particle size, solvent concentration and solvent-solid ratio of extraction (Chirinos et al., 2007, Pinelo et al., 2007). Aqueous acetone (typically between 50-70% v/v) has shown to be the most effective solvent for the extraction of different phenolic sub-groups from a range of plant materials, such as proanthocyanidins from hops (Gadon et al., 2019a). However recently more environmentally friendly, sustainable non-toxic solvents such as ethanol are preferred as they are more suited to produce food grade extracts. Other ‘green’ solvents that have been evaluated for the extraction of phenolics include propylene glycol (Myo and Khat-Udomkiri, 2022), glycerol (Huamán-Castilla et al., 2020) and deep eutectic solvents (DES) (Lakka et al., 2019).

The impact of extraction conditions on the phenolic composition of extracts as well as the growing trends towards using 'green' techniques suitable for the food and beverages industries, is discussed in more detail in Chapter 3. Techniques for the purification of phenolics from crude extracts is discussed in Chapter 5.

1.2.5-Analytical methods for measuring phenolic content

Quantifying phenolic compounds in plant extracts, including hops, remains difficult due to the large number of individual compounds present and their different chemical structures. The most abundant phenolics in hop cones are prenylflavonoids, proanthocyanidins and flavonols which vary in terms of molecular weight and polarity making it difficult to accurately measure all phenolic groups with one method (Knez Hrnčič et al., 2019). Currently, a range of analytical techniques are employed for the quantitation of polyphenols in hop material, ranging from non-specific semi-quantitative spectrophotometric assays to more complex chromatographic techniques (Önder et al., 2013).

1.2.5.1-Spectrophotometric techniques

Spectrophotometric assays are commonly used to measure the phenolic content of extracts as they are simple, cheap and quick. These methods provide a summative semi-quantitative measurement of phenolics and often serve as a starting point for phenolic evaluation before more specific analysis. Assays have been developed to measure either the total phenolic content of an extract, including all phenolic compounds, or to target specific phenolic sub-groups, such as total flavonoid content (Chlopicka et al., 2012) or proanthocyanidin content using the acid-butanol assay (Gessner and Steiner, 2005). The phenolic standards used for quantitation vary depending on the assay but are typically cheap, widely available, non-toxic and provide an adequate working linear range. Many of these assays have been adapted to a 96-well plate format which allows for a higher throughput and lower reagent cost and waste (Sánchez-Rangel et al., 2013).

The Folin-Ciocalteau assay developed by Singleton et al. (1999), is the most widely used method for evaluating total phenolic content (TPC). It is based on the chemical reduction of the Folin-Ciocalteau reagent at a basic pH. Gallic acid is the most widely used standard however others such as caffeic acid have also been found to be suitable, depending on the plant material being analysed (David et al., 2015). The Folin-Ciocalteu assay is often used alongside other assays evaluating specific phenolic sub-groups such as total flavonol content. This allows for analysis of the relative proportions of different groups in relation to total phenolic content.

Despite its widespread use, the Folin-Ciocalteau assay lacks specificity as the reagents can also react with non-phenolic compounds such as sugars and ascorbic acid (Khoddami et al., 2013). To improve phenolic specificity, several modifications have been proposed to remove interfering compounds such as using SPE purification prior to analysis or by treating samples with hydrogen peroxide (Sánchez-Rangel et al., 2013). The accuracy of this assay can also be influenced by differences in how phenolic compounds react with the reagent, making the analysis highly dependent on the standard used as well as the phenolic composition of the extract (Bastola et al., 2017). For example, Ruiz-Ruiz et al. (2020) highlighted that the TPC of green tea was 1.2 times higher when expressed as epigallocatechin equivalents compared to gallic acid equivalents. Despite these limitations, spectrophotometric methods remain widely used for semi-quantifying a range of phenolic groups, including total phenolics, proanthocyanidins, and flavonoids.

The analysis of proanthocyanidins presents challenges mainly due to the large differences of molecular weight. Non-specific spectrophotometric methods used for proanthocyanidin analysis include the DMAC assay (Payne et al., 2010), the vanillin assay (Deshpande and Cheryan, 1985), and the acid butanol assay. However, challenges remain in finding suitable standards for semi-quantitation. For example the acid butanol assay measures condensed

tannins by oxidative cleavage of proanthocyanidins under acidic conditions, producing anthocyanins that are measured spectrophotometrically at 550 nm (Gessner and Steiner, 2005). However the absorbance intensity of the anthocyanin varies depending on proanthocyanidin degree of polymerisation and plant material (Li et al., 2010), which can limit the accuracy of semi-quantitative analysis. Li et al. (2010) recommended to prepare standards from the plant material of interest and used Amberlite XAD7HP adsorption resin to develop a standard for the semi-quantitation of proanthocyanidins in apple extracts.

1.2.5.2-Chromatographic techniques

To analyse the phenolic composition of complex mixtures, chromatographic techniques are most widely used, typically coupled with UV or MS detection. Chromatographic techniques coupled with UV/Vis and MS/MS for the 'tentative' identification and quantitation phenolics are discussed in more detail in Chapter 2.

In hops, proanthocyanidins are present as dimers and trimers but they have also been reported up to 100 KDa in Saaz cones (Gadon et al., 2019a). Low molecular weight proanthocyanidins can be easily separate and detected using reverse-phase liquid chromatography and (-) MS, however high molecular weight (HMW) proanthocyanidins (>20 DOP) suffer from decreasing ionisation (Anke et al., 2008). A variety of different approaches have been taken to analyse HMW PAC's such as thiolysis combined with LC-MS/MS as well as analytical ultracentrifugation (Taylor et al., 2003, Gadon et al., 2019b, Yanagida et al., 2003). However the availability of reference standards for proanthocyanidins is poor which limits identification and accurate quantitation of these compounds (Kelm et al., 2005).

1.2.6-Phenolic antioxidant activity

The functional properties of hop phenolics are primarily related to their antioxidant capacity. Antioxidant activity refers to a molecule's ability to slow or inhibit oxidation, either by scavenging reactive oxygen species (ROS) or suppressing their formation. Antioxidants that

prevent or slow the initial formation of free radicals are sometimes termed ‘preventive’ (Proestos and Komaitis, 2009). This includes different mechanisms such as chelating metal ions which could otherwise catalyse the formation of hydroxyl radicals, or promoting the synthesis of inhibitory enzymes (Kumar and Pandey, 2013, Keskin et al., 2019). Radical scavengers neutralise ROS directly by either electron transfer (ET) or hydrogen atom transfer (HAT) mechanisms, and most antioxidant assays are categorised on this basis.

The antioxidant activity of phenolic compounds is closely tied to its structure. Phenolics act as strong antioxidants due to their hydroxyl groups which enable them to donate hydrogen atoms or electrons (Vuolo et al., 2019). Other structural features can further influence antioxidant properties. Glycosylation can decrease antioxidant activity (Xie et al., 2022), whilst polymerisation of flavanols to proanthocyanidins has shown to increase activity (up to 10 mean degrees of polymerisation) (Zhou et al., 2014). The influence of structure on the radical scavenging activities of phenolics is discussed in more detail in Chapters 5 and 7.

Proestos and Komaitis (2009) evaluated the antioxidant properties of ethanolic hop cone extracts and found comparable antioxidant activity to butylated hydroxytoluene and ascorbic acid which are routinely used by the food and beverage industries. Similarly, Wang et al. (2014) evaluated the antioxidant activity of an SP850 purified hop cone extract composed of proanthocyanidins, flavonol glycosides and chlorogenic acids which was found to have higher activity compared to a purified tea extract.

1.2.6.1-Methods for evaluating the antioxidant capacity of phenolic extracts

Radical scavenging activity assays are generally classified based on their reaction mechanism into electron transfer (ET) and hydrogen atom transfer (HAT). The most widely used electron transfer techniques are the ferric reducing antioxidant power (FRAP), ABTS and DPPH due to their convenient nature and wide applicability. For most of these assays the same principle is involved where a synthetic-coloured radical is produced, and the ability of a biological

sample to scavenge the radical or reduce redox-active compound is monitored by spectrophotometry. There is a lack of consensus on the validity of these methods possibly a result of problems associated with using one-dimensional methods to evaluate multifunctional foods and various improvements have been suggested (Frankel and Meyer, 2000). Due to the nature of the different antioxidant assays, they will generate different values depending on the assay and therefore are often performed in tandem (Floegel et al., 2011). For example, ET and HAT assays FRAP and ORAC respectively were deemed suitable for evaluating brewing raw materials on antioxidant activity of resulting beers (Wannenmacher et al., 2019).

HAT-based assays typically involve peroxyl radicals as oxidants which play a role in lipid oxidation in food and biological systems. The ORAC method has emerged as the HAT-based method of choice to quantify the peroxyl radical scavenging capacity of a sample. The ORAC assay, initially developed by Ou et al. (2001) measures the area under a decay curve of a fluorescent probe, which is typically expressed as Trolox equivalents. B-phycoerythrin was previously used as the fluorescent probe, however fluorescein has since become standard as it doesn't react with antioxidant extracts, and has better photostability (Ou et al., 2001).

The hydroxyl radical antioxidant capacity (HORAC) assays measures hydroxyl radical scavenging activity also via measuring the area under a decay curve of a fluorescent probe (Ou et al., 2002). Transition metals such as copper sulphate (Van Hoyweghen et al., 2010) and cobalt chloride (Harada et al., 2011) react with hydrogen peroxide forming hydroxyl oxidants via the Fenton reaction. Whereas most antioxidant assays use Trolox as a standard, the HORAC assay uses gallic acid as Trolox interacts poorly with the assay when cobalt chloride is used as the metal source. Xanthohumol extracts such as XanthoFlav^{tm} exhibit a peroxyl radical capacity 5-10* that of Trolox, however a hydroxyl radical scavenging capacity equivalent to 60 Trolox which was 20-30* that of an oligomeric proanthocyanidin extract and quercetin (Van Hoyweghen et al., 2010). The ORAC and HORAC assays can also be used to evaluate

lipophilic phenolic compounds and extracts using aqueous acetone and beta cyclodextrin as a solubility enhancer (Huang et al., 2002a).

1.2.7-Added value applications for plant phenolics

1.2.7.1-Beer flavour stability

Oxidation is a key factor in beer ageing, primarily driven by ROS generated through various pathways such as the Fenton and Haber-weiss reactions (Andersen and Skibsted, 1998). These processes lead to the formation of undesirable compounds such as Strecker aldehydes and lipid oxidation aldehydes like trans-2-nonenal which negatively impact the sensory qualities of the beer (Mertens et al., 2022). Phenolics from plant sources have been investigated for their ability to neutralise ROS and chelate metal ions and therefore improve the 'freshness' and flavour stability of beer (Mertens et al., 2021). As such plant phenolic extracts have been developed and are commercially available to improve beer stability. An example is Brewtan B, a plant derived gallotannin extract. This is especially important for light lagers or beers produced with reduced hop products e.g. tetra-iso-alpha-acids which contain no polyphenols (Jaskula-Goiris et al., 2014).

In light of this, phenolics from hop co-products have also been assessed to improve beer stability. Jaskula-Goiris et al. (2014) found that an extract from Saaz-CO_2R, primarily composed of flavonol glycosides, prenylflavonoids and procyanidins enhanced the mouthfeel and flavour stability of beer. Similarly, Jelínek et al. (2014) investigated the addition of phenolics from T45R which increased the antioxidant capacity of the final beers. A possible drawback of using hop processing residue is a high nitrate content which can lead to negative health effects via the formation of carcinogenic nitrosamines (Bamforth, 2008). Forster et al. (1995) found nitrate levels in beer increased with use of bracteole fraction; however follow up work by Jaskula-Goiris et al. (2014) found no difference in nitrate levels of spent hop

treated beer compared to T90P treated beer which were well within the maximum levels permitted in drinking water (50 mg/L) (Schuddeboom, 1993).

1.2.7.2-Beer colloidal stability

Generally, beer is intended to be served without visible haze which can be caused by insoluble particles. Fining agents, like isinglass are commonly used in brewing to remove these particles by aggregating them to larger particles which then settle out rapidly. Isinglass finings are composed of purified collagen extracted from the swim bladders of tropical fish and are the most widely used for cask ales to produce a compact sediment of both chill hazes and yeast cells (Freeman et al., 2003). However due to its origin isinglass is not suitable for vegetarians or kosher certification which limits its use, when considering trends suggesting a quarter of UK people will be vegetarian by 2025 (Брусницына, 2020).

As a result of these concerns, alternative natural sources have been explored to rival conventional fining agents such as isinglass and gelatin (Ham, 2023). Proanthocyanidins extracted from Saaz CO_2R have been evaluated for their fining potential in beer and have shown comparable activity to isinglass performance (Linforth et al., 2015). They produce a compact sediment from yeast flocculation important to avoid beer losses and seem to be effective in reducing hazes at both ale (12°C) and lager (4°C) temperatures. A natural hop fining agent with demonstrated functionality may have commercial value, especially in the UK where cask beer is popular (Linforth et al., 2015). Other benefits include reduced costs of downstream separation methods e.g., filtration, centrifugation. As a natural product occurring in beer, hop tannin use is also not constrained by legislative barriers such as the 'Reinheitsgebot'.

1.2.7.3-Pharmaceuticals and nutraceuticals

The lower incidence of diseases in East Asian populations, compared to western countries, has been partially attributed to diets rich in phenolic-containing foods such as soybeans (Fraga et al., 2019). Similarly the reduced rate of coronary heart disease among wine drinkers

has been linked to the intake of phenolics (Renaud and de Lorgeril, 1992). A growing body of research has highlighted the potential health benefits of hop polyphenols (Zanoli and Zavatti, 2008, Stevens and Page, 2004).

Proanthocyanidins exhibit a range of biological activity and benefits to human health, e.g. cardiovascular and neurodegenerative disease prevention, which seem to be related to their free radical scavenging activity and antioxidant properties (Bagchi et al., 2000, Packer et al., 1999). Proanthocyanidins, especially B2, have been shown to inhibit nitric oxide synthase (NOS), which may be explained by their protein binding activity (Kobuchi et al., 1999, Stevens et al., 2002). Dental studies have shown that high molecular weight polyphenols from hop bracts found in high concentrations in T45R also exhibit cavity-prevention activity and dental-plaque regrowth inhibition (Kurumatani et al., 2005, Shinada et al., 2007). They inhibit cellular adherence of *Strerptococcus sobrinus* and glucosyltransferase action at concentrations much lower than alternative e.g., green tea leaves (Xi et al., 2009). This suggests spent hop bract material could have value in the health and dentistry fields, however the chemical structure of the active compound hasn't been fully clarified (Tagashira et al., 1997). There is also evidence that polyphenols rich spent hops possess anti-inflammatory properties via decreasing the expression of inflammatory cytokines (Caban et al., 2020).

1.2.7.4-Cosmetics

The growing interest in natural ingredients for cosmetic formulations has led to increased exploration of phenolic compounds from agri-food residues. Grape derived by-products have received most attention, resulting in numerous patents for their use (Nunes et al., 2017). This is largely due to their anti-inflammatory and antioxidant activity which can protect the skin from disease and premature ageing (Ratz-Łyko et al., 2015). More recently phenolics from hop co-products have shown similar promise in the area (Alves et al., 2021, Paredes-Ramos et al., 2022, Pereira et al., 2022). Censi et al. (2021) adopted antioxidant and phenolic assays

as well as cytotoxicity and mitochondrial analysis of human keratinocytes identifying spent hops and yeast as the most effective brewing by-products for skincare applications Several patents have been filed for the use of hop phenolics in cosmetic.

EP1854446B1-2006 explored aqueous ethanolic extracts (10% v/v) from hop leaves and stem tissue for their antiallergic properties. This activity is attributed to kaempferol glycosides such as 3-O-glucoside, 3-O-rutinoside and 3-O-malonyl glucoside which inhibit histamine release. Another patent, **CZ283179B6-1995** focused on obtaining proteins and tannins from hop CO_2 extract residue via ultrafiltration for prophylactic cosmetics. Additionally, hop CO_2 extract residue has also shown anti-bacterial properties against *P.acnes*, a bacteria associated with acne vulgaris (Weber et al., 2019, Yamaguchi et al., 2009). Hop phenolics could offer a safer, natural alternative to conventional treatments like benzoyl peroxide, which can generate free radicals in skin leading to irritation and premature ageing (Kennedy et al., 1995).

1.3-Overview of thesis content

Hop co-products from the cultivation and processing of hop cones are produced in substantial quantities and are a rich source of phenolic compounds that could be similarly exploited for their antioxidant properties. The objective of this thesis is to evaluate hop co-products as source materials for phenolic valorisation. It is aimed to characterise and compare hop co-products from different streams to identify those most suited for exploitation. It is further aimed to investigate different extraction and purification strategies suited to the food and beverage industries for the generation of extracts with high phenolic purity and antioxidant activity and to support the development of commercial hop phenolic extracts.

Chapter 2-General Materials and Methods

2.1-Materials

2.1.1-Chemicals

Ammonium iron sulfate dodecahydrate, butanol, orthophosphoric acid, Folin-Ciocalteu phenol reagent, iron (III) chloride hexahydrate, methanol (LC grade), ethanol, hydrochloric acid, trolox, 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2'-Azobis (2-amidinopropane) dihydrochloride (AAPH), sodium phosphate buffer solution (pH 7.4, 0.2 M), fluorescein sodium salt, anhydrous sodium acetate, and 2,4,6-tri(2-pyridyl)-1,3,5-triazine (TPTZ) were obtained from Sigma Aldrich (St. Louis, USA). Formic acid (LC-MS grade) was obtained from Fisher Scientific (Loughborough, UK). Type 1 LC-MS grade water was produced using a Lab Pro PuraQ water purification system (Scientific Laboratory Supplies, Nottingham, UK). Authentic phenolic standards for chromatographic and spectrophotometric quantitative analysis were sourced as detailed in section 2.2.6.2. International calibration extract (ICE-3) was sourced from Labor Veritas (Zürich, Switzerland).

2.1.2-Plant materials

Hop and non-hop materials analysed in this study were sourced according to Table 2.1. The hop co-products included processing residues and hop leaves for commercially significant varieties, whilst hop pellets were included to assess the impact of processing mechanism on the phenolic content of the residue. All hop co-products were sourced from commercial suppliers, reflecting industry-scale co-products rather than laboratory-prepared samples. Non-hop materials (10-15, Table 2.1) were sourced to serve as benchmarks against which to compare the phenolic content of hop co-products.

Table 2.1: Characteristics and sourcing of hop co-products and other plant materials evaluated in this study.

	Material	Variety	Growing Region	Acronym	Moisture (%)	Supplier	Notes
1	Hop CO_2 Residue	Hallertau Blanc	Germany	CO_2R-HB	9.05 ± 0.05	HVG	-
2	Hop CO_2 Residue	Herkules	Germany	CO_2R-HERK	9.18 ± 0.23	Hopsteiner	-
3	Hop T45 Residue	Tradition	Germany	T45R-TRAD	8.56 ± 0.14	Barth Haas	-
4	Hop T45 residue	Saaz	Czech Republic	T45R-SAAZ-CR	8.56 ± 0.11	Hopsteiner	-
5	Hop T45 residue	Saaz	Hallertau	T45R-SAAZ-HT	7.04 ± 0.42	Hopsteiner	-
6	Hop T45 pellet	Saaz	Hallertau	T45P-SAAZ-HT	8.98 ± 0.25	Hopsteiner	-
7	Hop T90 pellet	Saaz	-	T90P-SAAZ	8.22 ± 0.22	Hopsteiner	-
8	Hop Leaf	Southern Aroma	South Africa (George)	LEAF-SA	11.54 ± 0.35	SAB Hop Farms	-
9	Hop Leaf	Southern Passion	South Africa (George)	LEAF-SP	9.59 ± 0.06	SAB Hop Farms	-
10	Seabuckthorn Pomace	NA	NA	SBT-POM	6.67 ± 0.37	Cornish Seaberry Company	Dried at 50-60 °C for 24 h.
11	Ground Cinnamon (*C. cassia)*	NA	NA	CIN	6.75 ± 0.15	K2B LTD	-
12	Tick Tock Tea (Rooibos)	NA	NA	TEA	7.95 ± 0.04	Tesco	-
13	Cranberry	NA	NA	CB-1	2.51 ± 0.52	K2B LTD	Freeze dried.
14	Cranberry	NA	NA	CB-2	2.27 ± 0.92	HEALTH FOODS	Freeze dried.
15	Blackcurrant	NA	NA	BC	3.15 ± 0.15	HEALTH FOODS	Freeze dried.

Moisture analysis represents averages ± standard deviation of triplicate measurements analysed according to 2.2.2.

Supplier headquarters: HVG-Wolnzach, Germany. Hopsteiner-Mainburg, Germany. Barth Haas-Nuremberg, Germany. SAB Hop Farms-George, South Africa. Cornish Seaberry Company-Cornwall, UK. K2B LTD-Sleaford, UK. Tesco-Welwyn Garden City, UK. HEALTH FOODS-Abergele, UK.

To further investigate hop leaves as a source of phenolics, hop leaves and their respective cones were sourced from Hopsteiner. These samples were collected from a commercial farm in the Yakima Valley, Washington and included leaf material collected over two crop years and three developmental stages for the varieties Calypso, Cascade and Contessa. Additional details on these samples are provided in Chapters 6.

2.2-Methods

2.2.1-Extraction of phenolics from plant materials

Plant material (>100 g) was homogenised and milled with a De'longhi Blade KG49 grinder (Treviso, Italy) at speed 12 for 25 s until a fine consistency was achieved. For extraction, 10 g

milled plant material was mixed with 100 ml of 50% aqueous ethanol (v/v) (E50) and agitated using a Stuart Roller Shaker (Cole-Palmer, Staffordshire, UK) at 60 rpm for 15 min. The extract was then filtered through Whatman grade 1 filter paper (Maidstone, UK) using vacuum filtration. The filtered solution was then transferred to a 50 ml Falcon tube ensuring minimal headspace was present before being chilled overnight at 4 °C. The extract was then centrifuged for 10 min at 4000 rpm using a Thermo Scientific Heraeus Megafuge 16 Centrifuge (Waltham, USA), with the supernatant stored at -80 °C for subsequent analysis. Extractions were performed in triplicate for each material unless otherwise stated.

These extraction conditions were selected based on phenolic extraction optimisation experiments using CO_2R-HERK which is presented in Chapter 3. These conditions were used for all extractions in Chapters 4-7.

2.2.2-Moisture content of hop materials

The moisture content of plant materials was determined using an Ohaus MB120 Moisture Analyser (Parsippany, USA) where material (1-5 g) was dried to a constant weight at 105 °C. The moisture content of materials was used to standardise phenolic concentrations to dry material (DM).

2.2.3-Dry Material Yield

Dry material yield was determined using a Thermo Scientific Savant SpeedVac SPD300 centrifugal evaporator. A 200 µl aliquot of extract was dried at 45 °C for two hours at 1.4 Torr. The resulting dry material, which comprised all non-volatile components under these conditions, was expressed as mg dry material extracted/g dry matter (DM).

2.2.4-Spectrophotometric assays for semi-quantitation of phenolics

The Folin-Ciocalteu assay is a commonly used colorimetric assay to semi-quantify phenolic contents whilst the acid butanol assay measures condensed tannins by oxidative cleavage of

proanthocyanidins under acidic conditions yielding anthocyanins which are measured spectrophotometrically (Gessner and Steiner, 2005).

2.2.4.1-Folin-Ciocalteu assay for the determination of total phenolic content (TPC)

The total phenolic content (TPC) of extracts was determined using a method modified from Singleton et al. (1999). Folin-Ciocalteu phenol reagent was diluted 1: 10 with deionised water. For analysis 1500 µl dilute Folin-Ciocalteu reagent was added to a microcuvette containing 150 µl sample, standard or blank solution. These were then stored in the dark for 2 h before UV/Vis absorbance was measured at 750 nm using a Thermo Scientific Genesys 140 Vis spectrophotometer against a reverse osmosis (RO) water negative control. Caffeic acid was used to produce a six-point standard curve (0.01-0.5 mg/ml E50), and results were expressed as mg caffeic acid equivalent (CAE) per g of dry material (mg CAE/g DM). For TPC semi-quantitation, caffeic acid was selected as reported previously by David et al. (2015) to determine the phenolic content of tea. To convert data to mg/g DM, the following formula was used:

$$\text{mg/g DM} = \frac{(\text{mg CAE/ml} \times \text{dilution factor} \times \text{liquid: solid ratio of extraction})}{(100 - \text{moisture content of plant material})/100}$$

Liquid: solid ratio (10) based on extractions at 100 mg plant material/ml E50.

2.2.4.2-Acid Butanol Assay for the determination of proanthocyanidin content (PAC)

The proanthocyanidin content (PAC) of extracts was determined using a method modified from Gadon et al. (2019a). An acid butanol solution was prepared from 5 ml HCl (37%) with 95 ml n-butanol. 2% ferric ammonium sulfate solution was prepared by dissolving 1 g of ferric ammonium sulfate in 50 ml of 2 M HCl. 3 ml of acid butanol solution was added to a 10 ml pyrex screw-capped glass tube with 25 µl sample solution and 100 µl iron reagent. Tubes were vortexed and heated at 90 °C for 30 min using a Thermo Scientific multi-block heater before being cooled for 30 min. UV/Vis absorbance was measured at 550 nm using a Thermo Scientific Genesys 140 Vis spectrophotometer against an acid butanol solution negative

control. Procyanidin B3 (P-B3) was used to produce a six-point standard curve (1-6 mg/ml), and results were expressed as mg P-B3 equivalent per g of DM (mg PB3E/g DM). For PAC semi-quantitation, a B-type procyanidin was used as these are the main proanthocyanidins found in hops (Li and Deinzer, 2009).

2.2.5-Antioxidant Assays

In this study, antioxidant assays were used to comprehensively evaluate different mechanisms of radical scavenging activity. The ORAC assay evaluates peroxyl radical scavenging via hydrogen atom transfer (HAT) whilst the FRAP and DPPH assays evaluate single electron transfer (ET) (Moharram and Youssef, 2014). These techniques were selected for this study as they are well established, high-throughput techniques relevant to the food and beverage industries (Wannenmacher et al., 2019).

2.2.5.1-Ferric Reducing Antioxidant Power (FRAP)

FRAP antioxidant activity was determined using a method modified from Xiao et al. (2020). An acetic acid buffer solution was prepared by dissolving 455.30 mg of anhydrous sodium acetate with ultrapure (UP) water. The FRAP working solution was prepared by mixing 5 ml 2,4,6-Tris(2-pyridyl)-s-triazine (TPTZ) solution (3.12 mg/ml in 40 mM HCl), 5 ml ferric chloride hexahydrate solution (5.42 mg/ml in UP water) and 50 ml acetic acid buffer (pH 3.6). In a 96-well plate, 180 µl of working FRAP solution was added using a multi-channel pipette to each well containing 20 µl of sample, standard or negative control solution. The plate was shaken for 30 s (double orbital) and incubated at 25 °C for 30 min using a FLUOstar Omega microplate reader (Ortenberg, Germany). Absorbance was measured at 593 nm against a negative control. Trolox was used to produce an 8-point standard curve (0.01-0.18 mg/ml) and results expressed as mg trolox equivalent per g of dry material (mg TE/g DM). The linearity of the FRAP assay for trolox and leaf extracts was evaluated to ensure accurate TE values could be obtained from analysis of one concentration. Excellent linearity was obtained for Trolox and

hop leaf extract up to an absorbance of 1.75 at 593nm (Appendix 2-Figures 1 and 2). The FLUOstar plate reader was operated using Voyager software (v2.05) and data was acquired using MARS software (v5.10).

The FRAP assay originally developed by Benzie and Strain (1996) assumes that redox reactions are rapid and typically reach completion by around 5 mins. Whilst this may be adequate for fast reacting phenols, certain compounds such as quercetin and caffeic acid have been shown to react more slowly and require longer reaction times for detection (Pulido et al., 2000). Additionally, studies have demonstrated that aqueous ethanol (60% v/v) extracts of hop cones required longer reaction times in the FRAP assay compared to ethanol extracts (Proestos and Komaitis, 2009). To accommodate these slower-reacting phenolics, a 30 min reaction time was chosen, as suggested by Proestos and Komaitis (2009). Absorbance changes at 593 nm for hop leaf extracts and Trolox standard solution measured at 5 min intervals over 30 mins are presented in Appendix 2-Figure 3.

2.2.5.2-DPPH Radical Scavenging Activity (RSA)

DPPH radical scavenging activity (RSA %) was determined using a method modified from Sharma and Bhat (2009). DPPH (40 mg) was dissolved in 100 ml of ethanol to prepare a 400 µg/ml solution, which was protected from light using aluminium foil and sonicated for 2 h. Dilution adjustments of DPPH solution were made with ethanol to achieve an absorbance of 1 ± 0.005 at 517 nm for the negative control. Extracts were diluted with E50 to a volume of 3 ml for 6 concentrations and mixed with 1 ml DPPH solution. Solutions were vortexed (10 s) and left to stand for 30 min in a dark cupboard. UV/Vis absorbance was measured at 517 nm against an E50 negative control and RSA % was calculated using the formula:

$$\text{RSA } (\%) = 100 - \frac{(\text{Abs control} - \text{Abs sample})}{\text{Abs control}} \times 100$$

RSA % (y) was plotted against sample concentration (x) and linear regression was used to calculate the concentration of extract required for a 50% reduction of DPPH (IC50). Extract concentrations analysed were selected to ensure that 50% RSA was within the analysed range. Trolox was analysed at 6 concentrations (4.18 µg/ml-8.36 µg/ml) to calculate an IC50 which was used to generate Trolox equivalents which were expressed as mg Trolox equivalent per g of dry material (mg TE/g DM). The concentrations of extracts analysed for RSA % used to calculate Trolox equivalent IC50's are presented in Appendix 4-Figure 1, Appendix 5-Figure 2, Appendix 7-Figures 1 and 2.

2.2.5.3-Oxygen Radical Absorbance Capacity (ORAC)

The ORAC assay measures a samples peroxyl radical scavenging by quantifying the inhibition of fluorescence decay which corresponds to the area under curve (AUC). ORAC antioxidant activity was determined using a method modified from Huang et al. (2002b). A 0.2 M sodium phosphate buffer solution (PBS) (pH 7.4) was diluted with ultrapure (UP) water to 75 mM. Fluorescein stock solution was prepared by dissolving fluorescein sodium salt in PBS (0.2 M) at 1.97 mg/ml and stored at -20 °C. Fluorescein and AAPH working solutions were prepared daily in PBS (0.2 M) at 48 nM and 403 mM respectively and stored at 4 °C. Trolox standard solution was prepared in PBS (75 mM) for hydrophilic assays and in 50% aqueous acetone (v/v) for lipophilic assays at 0.5 mg/ml with 7 concentrations analysed to generate a standard curve (0.005-0.05 mg/ml).

For analysis, extracts were diluted in PBS (75 mM) or 50% aqueous acetone (v/v) (for lipophilic assays) and 20 µl sample, standard and negative control solutions were transferred to a black opaque 96 well plate (Corning Costar). 160 µl working fluorescein solution was transferred to each well using a multi-channel pipette and the plate was then shaken for 30 s (double orbital) and incubated at 37 °C for 20 min using a FLUOstar Omega microplate reader. Fluorescence measurements (Ex. 485 nm, Em. 520 nm) were taken every 30 s to monitor the fluorescence

stability. 20 µl AAPH solution was then injected using onboard injectors followed by a 10 s shake (double orbital). Fluorescence was measured every 60 s (Ex. 485 nm, Em. 520 nm) until the reaction was complete (<90 min) which was determined when the fluorescence reached the level of the negative control. ORAC Trolox equivalent antioxidant activity values were calculated using linear regression by calculating the net area under fluorescence decay curve (NAUC) relative to the Trolox standard and expressed as mg Trolox equivalent per g of dry material (mg TE/g DM). The FLUOstar plate reader was operated using Voyager software (v2.05) and data was acquired using MARS software (v5.10). Fluorescence decay curves for Trolox analysed at concentrations between 0.005-0.05 mg/ml are presented in Figure 2.1 whilst the corresponding linear standard curve is presented in Appendix 2-Figure 4. Figure 2.1 outlines how the NAUC corresponds to an increase in Trolox concentration.

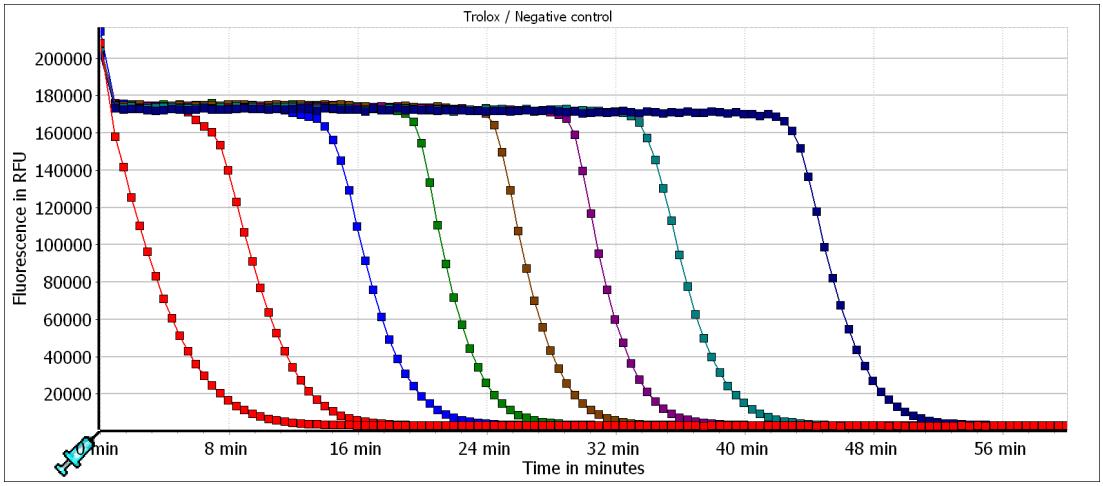


Figure 2.1: Fluorescence decay curves for Trolox analysed at concentrations of 0, 0.005, 0.0125, 0.01875, 0.025, 0.03125, 0.0375 and 0.05 mg/ml in PBS.

2.2.6-Chromatographic analysis of phenolics and hop resin components

2.2.6.1-Analysis of xanthohumol and bitter acids in hop extracts using HPLC-UV-DAD

The quantitation of xanthohumol, α and β-acids in hop extracts was performed using HPLC-UV-DAD following a method adapted from the European Brewing Convention (EBC) 7.15 standard (EBC, 2018). Analysis was carried out using a Waters (Milford, USA) 2695 separations module coupled with a Waters 2996 photodiode array detector, with data acquired using

Empower software. A reversed-phase column (Gemini C18 4.6 × 250mm, 5 µm; Phenomenex, Torrance, CA, USA) was used for separation with the column oven maintained at 30 ± 2 °C. Extracts were syringe filtered at 0.45 µm with an injection volume of 11 µl. A gradient elution was performed using a two-solvent system comprising mobile phase A (75% methanol, 24% water and 1% orthophosphoric acid v/v), and B (methanol). Flow rate was 0.7 ml/min throughout. A linear gradient from 100% A to 45: 55 (A: B) was run over 40 min, followed by a return to 100% A over 1 min which was held for 4 min to re-equilibrate the column.

UV/Vis spectra were recorded in the range of 240-400 nm with quantification calculated from peak areas at the optimal wavelengths specified in Table 2.2. The wavelength chosen for each analyte was selected based on optimal peak resolution and signal intensity. 6-point standard curves were prepared using ICE-3 (0.0049-4.86 mg/ml) for α and β-acids, and xanthohumol (0.0022-0.87 mg/ml) dissolved in methanol. The analytical run order was randomised, and each extract analysed in triplicate unless otherwise stated. A representative chromatogram is provided in Figure 2.2.

Table 2.2: Chromatographic details used for HPLC-DAD quantitation of xanthohumol and bitter resins in hop extracts.

Analyte	Retention Time (min)	Optimal Wavelength (nm)
Xanthohumol	12.39	370
Alpha 1 (Cohumulone)	24.22	270
Alpha 2 (Ad/Humulone)	27.81	270
Beta 1 (Colupulone)	34.59	340
Beta 2 (Ad/Lupulone)	38.40	340

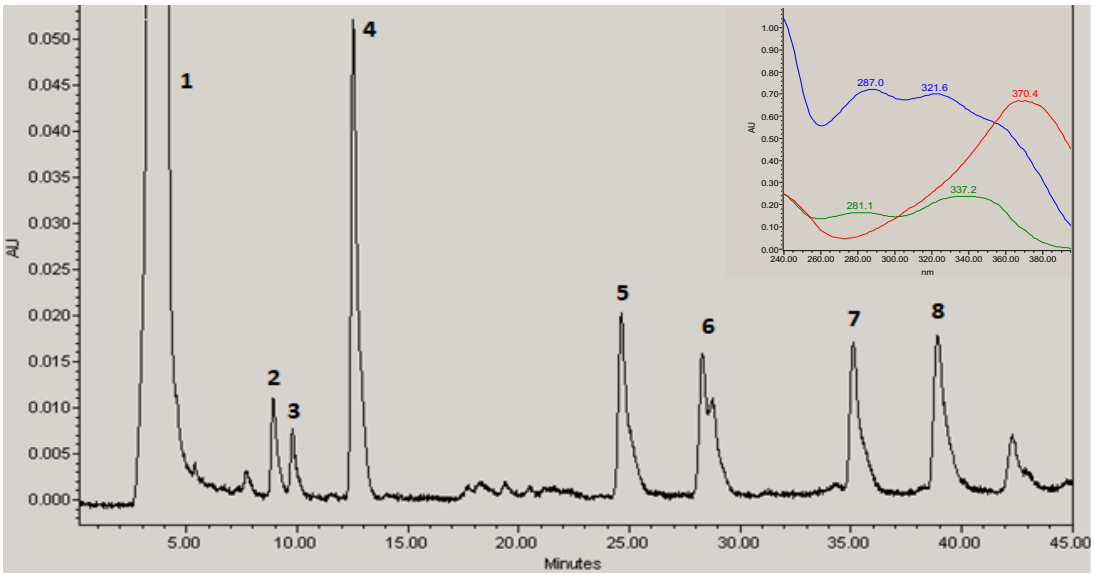


Figure 2.2: LC-DAD chromatogram of a hop leaf extract from Cascade (harvest 2021) analysed at 100mg/ml E50.

1-compounds not retained by reversed-phase column, **2/3**-unidentified peaks, **4**-xanthohumol, **5**-cohumulone, **6**-ad/humulone, **7**-colupulone, **8**-ad/lupulone.

UV spectra of hop standards (xanthohumol-red, Ad/humulone-blue, Ad/lupulone-green) used for compound identification is outlined in top right.

2.2.6.2-Phenolic identification and quantitation using LC-ESI-qTOF-MS/MS

LC-ESI-qTOF-MS/MS was selected for the analysis of low molecular weight phenolics due to the higher sensitivity of MS compared to DAD detection. MS also provides better resolution through *m/z* filtering which is particularly important when investigating crude extracts where co-elution of compounds occurs. For phenolic compound identification this method enables accurate mass determination using a qTOF and allows for the acquisition of MS/MS spectra to further confirm structural identities.

LC-ESI-qTOF-MS/MS analysis was performed using an Agilent HPLC 1260 II system coupled with a 6546 tandem quadrupole-Time of Flight mass spectrometer (Agilent Technologies, Cheadle, UK). Separation was performed with a Kinetex Biphenyl column (1.7 µm, 100 x 2.1mm; Phenomenex, Macclesfield, UK) at 40 ± 0.8 °C. A gradient elution was performed using a two-solvent system with mobile phase A (Type 1 water with 0.1% formic acid, v/v) and B (methanol with 0.1% formic acid, v/v), using a flow rate of 0.3ml/min. The 17 min analytical

gradient started at 5% B, was held for 2 min, raised to 19% over 6 min, then to 82.5% over another 9 min. The column was washed at 95% B, returned to starting conditions and re-equilibrated for a total run time of 25 min. (-) Electrospray ionisation (ESI) was performed between 50-1700 m/z, with drying gas and sheath gas temperatures of 320 °C and 350 °C respectively, and flow rates of 8 and 11 L/min, respectively. The nebulizer was set at 35 psi whilst VCap and nozzle voltages were 3500 V and 1000 V respectively, with fragmenter, skimmer and octopole RF voltages set at 110, 65 and 750 V respectively.

2.2.6.2.1-Phenolic compounds reference library

2.2.6.2.1.1-Standard compounds

To develop a phenolic reference library, 62 phenolic reference standards were analysed to determine retention time (RT) and mass spectra. These standards were selected based on availability and their reported abundance in hop cones (Schmidt and Biendl, 2023b), leaves (Choi et al., 2018), and processing residues (Luzak et al., 2016). For preparation, 1-1.5 mg of each standard was solubilised in 1-1.5 ml 50% aqueous ethanol (v/v) to produce 1 mg/ml solutions. 100 µl aliquots were dried using a SpeedVac SPD300 centrifugal evaporator and stored at the recommended temperature until analysis. For analysis 0.1 mg portions were re-solubilised in E50 to 0.01 mg/ml and diluted with E50 to 0.001 mg/ml. Internal standard (IS) candidates were selected if they were not present in the 'representative' extracts and had similar abundance to the main phenolic targets at the same concentration. The reference standards used in this study are presented in Table 2.3.

Table 2.3: Phenolic reference standards used in the study.

No.	Standard	Purity (%)	RT (min)	IS candidate	Supplier
1	Luteolin	≥ 98	14.49	-	Cayman Chemical Company (Ann Arbor, USA).
2	Kaempferol 3-O-galactoside	≥ 98	13.06	-	
3	Quercetin 3-O-sophoroside	≥ 98	11.68	-	
4	5-O-Feruloylquinic acid	≥ 98	9.06	-	
5	8-Prenylnaringenin	≥ 98	16.90	-	
6	6-Prenylnaringenin	≥ 95	17.47	-	
7	Procyanidin B1	≥ 98	6.81	-	
8	Procyanidin B2	≥ 98	9.77	-	
9	Procyanidin B3	≥ 98	7.15	-	
10	Procyanidin A1	≥ 99	11.61	-	
11	Procyanidin A2	≥ 95	12.25	✓	
12	Procyanidin C1	≥ 98	11.09	-	
13	Catechin	≥ 99	6.36	-	
14	Epicatechin	≥ 90	9.56	-	
15	3,4-Dicaffeoylquinic acid	≥ 98	12.51	✓	
16	4,5-Dicaffeoylquinic acid	≥ 98	13.24	-	
17	Cinnamtannin B1	≥ 85	10.63	✓	
18	Hesperidin	≥ 90	14.18	✓	
19	Hesperetin	≥ 98	15.51	✓	
20	Quercetin	≥ 95	13.98	-	
21	Taxifolin	≥ 95	11.81	-	
22	Quercetin 3-O-glucoside	≥ 98	12.58	-	Sigma Andrich (St. Louis, USA).
23	Quercetin 3-O-malonyl glucoside	≥ 85	12.93	-	
24	Kaempferol 3-O-rutinoside	≥ 98	13.15	-	
25	Neochlorogenic acid	≥ 98	4.77	-	
26	Chlorogenic acid	≥ 95	8.22	-	
27	4-Hydroxybenzoic acid	99	4.50	-	
28	Vanillic acid	≥ 97	7.91	✓	
29	p-Coumaric acid	≥ 98	10.36	-	
30	trans-3-Hydroxycinnamic acid	99	11.34	-	
31	3-Methylcatechol	98	6.76	✓	
32	4-Methylcatechol	≥ 95	7.01	✓	
33	4-Hydroxyphenylacetic acid	≥ 98	5.86	✓	
34	Benzoic acid	≥ 99.5	11.56	✓	
35	Sinapic acid	≥ 98	12.60	-	
36	trans-Cinnamic acid	≥ 99	14.21	✓	
37	Caffeic acid	≥ 98	7.12	-	
38	Phloroglucinol	99%	1.56	-	
39	Naringenin	≥ 95	14.87	-	
40	Phloridzin	99%	13.11	-	
41	Resveratrol	≥ 99	12.90	✓	

42	Ethyl gallate	≥ 96	10.00	✓	
43	Genistein	≥ 98	15.08	✓	
44	2,5-Dihydroxybenzoic acid	≥ 98	4.49	-	
45	3-Methoxycatechol	≥ 98	6.76	-	
46	Salicylic acid	≥ 99	4.87	-	
47	Quercetin 3-O-rutinoside	≥ 99	12.44	-	Extrasynthese (Genay, France).
48	Curcumin	≥ 97.5	17.35	-	
49	Kaempferol	≥ 99	14.81	-	
50	3-Hydroxyphenylacetic acid	99	6.76	✓	
51	Syringic acid	≥ 98	12.60	-	
52	Myricetin	98	12.98	✓	
53	Epicatechin gallate	95-99	11.81	✓	
54	Epigallocatechin gallate	95	10.31	-	Acros Organics (Geel, Belgium).
55	Daidzein	≥ 98	14.29	✓	
56	Vanillin	99	11.15	-	
57	Gallic acid	97.5	1.48	-	MP Biomedicals (Santa Ana, California).
58	Ferulic acid	99.0	11.88	-	
59	Protocatechuic acid	98	2.62	-	
60	Kaempferol 3-O-glucoside	98.5	12.58	-	HWI Group (Rülzheim, Germany).
61	Isoxanthohumol	≥ 98	16.36	-	LKT Laboratories (St. Paul, USA).
62	Xanthohumol	≥ 98	17.49	-	PhytoLab (Vestenbergsgreuth, Germany).

2.2.6.2.1.2-Structural elucidation using MS/MS in silico fragmentation.

Where reference standards were unavailable, four representative hop extracts were analysed, and candidate peaks for MS/MS putative structural elucidation were chosen based on peak height (>10,000), m/z, and retention time relative to known compounds. The representative extracts were E50 extracts of materials presented in section 2.3: CO_2R-HERK, CONE-Calypso and LEAF-SA and a blend of equal portions of extracts from leaves and cones sourced from Yakima. Total ion chromatograms (TIC's) for CO_2R-HERK, CONE-Calypso, LEAF-SA and the blend of leaf and cone extracts are presented in Figure 2.3.

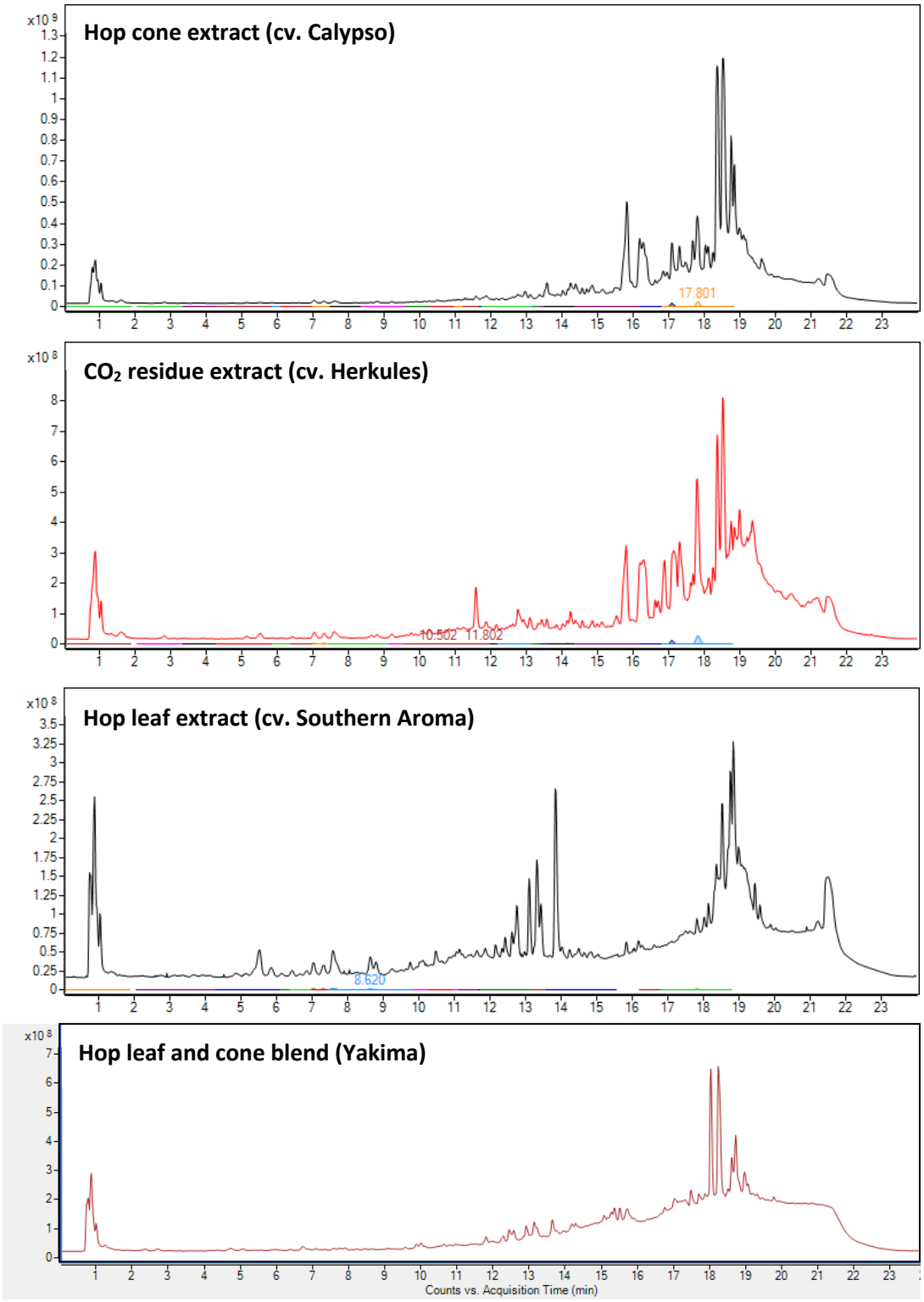


Figure 2.3: Total ion chromatograms for representative extracts; LEAF-SA, CO_2R-HERK, CONE-CAL and a blend of the Yakima leaf and cone extracts.

For the acquisition of spectra, auto MS-MS analysis ranging 50-1040 m/z was performed with MS1 and MS2 scan rates of 5 and 6 spectra/s respectively and collision energies were set at 10, 20 and 40 V. Spectra were exported to Sirius GUI software where Sirius and CSI:FingerID were used to produce match scores to different compounds based on fragmentation patterns. Peaks were annotated based on CSI match scores, SIRIUS scores and literature sources of key hop polyphenols.

In silico fragmentation annotation has been shown to be correct for more than 90% of phenolic compounds and is less time consuming and more systematic than manual interpretation of mass spectra (Mallmann et al., 2023, Fernandez-Ochoa et al., 2022, Dührkop et al., 2021). SIRIUS 4 software is an *in silico* tool for molecular formula annotation based on high-resolution isotope pattern analysis (Dührkop et al., 2019, Djoumbou Feunang et al., 2016). Sirius 4 is integrated with CSI:FingerID which predicts molecular structure from MS/MS fragmentation tree and spectrum (Shen et al., 2014, Dührkop et al., 2015). These are used to rank candidate structures based on fingerprint similarity using Bayesian network scoring (Ludwig et al., 2018). Generally, for structural annotation position 1 was used, however if similarly high scores were found for isomers of a compound, structure was annotated based on compound class. Features with a CSI score of < -150 or with conflicting SIRIUS and CSI scores were discarded. Sirius and CSI finger scores for structural annotation of features and structure rank used (position) are provided in Table 2.4. Features identified in the blend of hop leaf and cone extracts from Yakima, USA are only reported on in Chapters 6 and 7, as the MS/MS analysis of this extract took place after the conclusion of Chapter 4-6. MS2 fragmentation patterns and corresponding CSI:FingerID/position scores for kaempferol 3-O-(6''-malonyl-glucoside) (feature 29) are provided in Figure 2.4.

Table 2.4: Annotation of features from negative ion LC-MS/MS and reported Sirius and CSI finger scores.

Feature	RT (min)	m/z & Formula	Compound Class	Structural Annotation	Abundant Fragment Ions (MS2)	Fragments proportional to voltage (MS2)	SIRIUS Score, CSI & Position	Identity confirmed with standard	Extract identified
Chlorogenic acids									
1	5.24	353.09, **C16 H18 O9**	Caffeoylquinic acid	Caffeoylquinic acid isomer (CQA A)	191.05,135.05, 179.03	135.04	100, -3.87, 1	Neochlorogenic acid	LEAF
2	7.98	353.09, **C16 H18 O9**	Caffeoylquinic acid	Caffeoylquinic acid isomer (CQA B)	85.03, 209.03, 191.06, 179.03, 173.05, 135.04	85.03, 135.04	98.6, -21, 1	-	LEAF
3	6.93	337.09, **C16 H18 O8**	Coumaroylquinic acid	Coumaroylquinic acid isomer (COQA A)	163.04, 119.05, 191.06	119.05	100, -6, 1	-	Yakima blend
4	9.50	337.09, **C16 H18 O8**	Coumaroylquinic acid	Coumaroylquinic acid isomer (COQA B)	173.05, 119.05, 93.03, 163.04	93.03, 119.05	100, -17, 1	-	Yakima blend
5	10.08	337.09, **C16 H18 O8**	Coumaroylquinic acid	Coumaroylquinic acid isomer (COQA G)	173.05, 163.04, 119.05, 93.03	93.03, 119.05, 59.01	97.8, -22, 1	-	LEAF
6	10.75	337.09, **C16 H18 O8**	Coumaroylquinic acid	Coumaroylquinic acid isomer (COQA D)	191.06, 93.03	93.03, 191.05	99.3, -7.8, 1	-	LEAF
7	11.56	337.09, **C16 H18 O8**	Coumaroylquinic acid	Coumaroylquinic acid isomer (COQA E)	191.06, 93.03, 85.03	85.03, 93.03	100, -6, 1	-	Yakima blend
8	10.96	367.10, **C17 H20 O9**	Feruloylquinic acid	Feruloylquinic acid isomer (FQA D)	173.05, 193.05, 134.04, 93.03	134.04, 93.03	98.7, -10.7, 1	-	Yakima blend
9	11.22	367.10, **C17 H20 O9**	Feruloylquinic acid	Feruloylquinic acid isomer (FQA B)	173.05, 134.04, 93.03	77.04, 134.04, 93.03	79.42, -60.08, 1	-	LEAF
10	11.70	367.10, **C17 H20 O9**	Feruloylquinic acid	Feruloylquinic acid isomer (FQA G)	173.05, 193.05, 134.04, 93.03	134.04, 93.03, 117.03, 154.99	95, -13.5, 1	-	LEAF
Catechins and proanthocyanidins									
11	3.17	305.07, **C15 H14 O7**	Gallo Catechin	Gallo Catechin isomer (GC B)	125.02	125.02, 196.92	100, -2.41, 1	-	LEAF
12	6.671	305.07, **C15 H14 O7**	Gallo Catechin	Gallo Catechin isomer (GC A)	125.02	125.02	99.98, -97.67	-	LEAF
13	9.20	577.14, **C30 H26 O12**	B-type procyanidin	Procyanidin B dimer 1 (PBD 1)	125.02, 289.07, 425.09, 407.08	125.02	100, -2.75, 1	-	CONE
14	10.93	577.13, **C30 H26 O12**	B-type procyanidin	Procyanidin B dimer 2 (PBD 2)	125.02, 289.07, 161.02, 407.08	125.02	100, -2.55, 1	-	CONE
Flavonol glycosides									

15	10.86	609.15, **C27 H30 O16**	Kaempferol di glycoside	Kaempferol 3,7′-diglucoside (K-3,7-DIGLUC)	447.09, 283.03, 285.04, 446.09	283.03, 285.04	97.6, -13, 1	-	Yakima blend
16	11.02	755.20, **C33 H40 O20**	Kaempferol tri glycoside	Kaempferol -3-O-rhamnoside-7,4′-digalactoside (K-3-R-7,4-DIGALAC)	593.15, 755.20, 594.15, 285.04, 284.03, 283.02	285.04, 284.03, 286.04	94.49, -10.25, 1	-	Yakima blend
17	11.39	771.20, **C33 H40 O21**	Quercetin tri glycoside	Quercetin tri glycoside (Q-TG)	300.03, 301.03, 271.025	300.03, 301.03, 271.02	41.45, -32.87, 1	-	CONE
18	11.75	755.20, **C33 H40 O20**	Quercetin tri glycoside	Quercetin 3-O-rutinoside-O-rhamnoside (Manghaslin)	300.03, 300.04, 301.03	300.03, 301.03	93.8, -18, 1	-	Yakima blend
19	11.80	625.14, **C27 H30 O17**	Quercetin di glycoside	Quercetin 3-O-sophoroside (Q-SOP)	300.03, 301.03, 316.02, 271.03, 255.03	300.03, 301.03, 316.02, 271.03, 255.03	69.83, -50.32, 1	✓	LEAF
20	12.08	609.14, **C27 H30 O16**	Quercetin di glycoside	Quercetin 3-O-Neohesperidoside (Q-NEO)	300.03, 301.03, 271.02, 300.03	300.03, 301.03, 271.02	98.88, -9.8, 1	-	LEAF
21	12.30	739.20, **C33 H40 O19**	Kaempferol tri glycoside	Clitorin	284.03, 285.03	284.03, 285.04	98, -12, 1	-	Yakima blend
22	12.56	593.15, **C27 H30 O15**	Kaempferol di glycoside	Kaempferol di glycoside (K-DG)	284.03, 285.04, 384.99, 255.03, 284.04	284.03, 384.99	100, -39.70, 1	-	Yakima blend
23	12.72	463.09, **C21 H20 O12**	Quercetin mono glucoside	Quercetin 3-O-glucoside (Q-GLUC)	300.03, 301.03, 271.02, 255.03, 243.03	300.03, 301.03, 271.02, 255.03, 243.03	99.95, -27, 1	✓	CO_2R
24	12.98	593.15, **C27 H30 O15**	Kaempferol di glycoside	Kaempferol 3-O-neohesperidoside (K-NEO)	285.04, 284.03	285.04, 284.03, 286.04	90.4, -25, 2	-	Yakima blend
25	13.08	549.09, **C24 H22 O15**	Quercetin malonated glycoside	Quercetin 3-O-(6′′-malonyl-glucoside) isomer (Q-MG A)	300.03, 505.10, 506.10, 301.03,	300.03, 301.03, 271.03, 255.03	100, -2.63, 1	✓	LEAF
26	13.28	549.09, **C24 H22 O15**	Quercetin malonated glycoside	Quercetin 3-O-(6′′-malonyl-glucoside) isomer (Q-MG B)	505.10, 300.03, 506.10, 301.03	300.03, 271.02	100, -3.5, 1	-	LEAF
27	13.30	593.15, **C24 H22 O14**	Kaempferol di glycoside	Kaempferol 3-O-rutinoside (K-RUT)	285.04, 284.03, 286.04, 255.03	227.04, 229.05, 285.04, 284.03, 286.04, 255.03	98.55, -8.29, 1	✓	LEAF
28	13.43	447.09, **C21 H20 O11**	Kaempferol mono glycoside	Kaempferol mono glycoside	284.03, 255.03, 227.03, 285.04	255.03, 227.03,	100, -2.49, 1	kaempferol 3-O-glucoside	Yakima blend
29	13.65	533.09, **C24 H22 O14**	Kaempferol malonated glycoside	Kaempferol 3-O-(6′′-malonyl-glucoside) (K-MG)	284.03, 285.04	255.03, 227.04	99.83, -21.168, 1	-	Yakima blend
				Other					
30	0.87	191.05, **C7 H12 O6**	Cyclitol	L-Quinate	85.03, 59.01, 57.03, 111.01, 87.01	59.01, 57.03, 87.01	100, -20, 1	-	LEAF
31	0.98	175.02, **C6 H8 O6**	Butenolide	Ascorbate	87.01, 57.03, 69.03, 113.02	57.03, 59.01	100, -41.31, 1	-	LEAF
32	4.98	153.01, **C7 H6 O4**	Phenolic acid	2,6-dihydroxybenzoic acid	109.03, 108.99, 119.03, 62.98, 65.00	62.98, 65.00	97, -7.9, 1	-	Yakima blend

33	9.93	357.12, **C16 H22 O9**	Phenolic glycoside	3-(2-O-Beta-D-Glucopyranosyl-4-Methoxyphenyl)Propanoic Acid (Phenolic glycoside-A)	195.07, 59.01, 196.07, 136.05	59.01, 136.05	91.3, -40, 1	-	CO_2R
34	13.13	357.11, **C16 H22 O9**	Phloroglucinol glycoside	1-Butyrylphloroglucinol-Beta-D-Glucopyranoside (Phoroglucinol glycoside-C)	195.07, 196.07, 151.08, 219.07, 237.11	151.08, 152.08	99.88, -72.786, 1	-	CO_2R
35	15.66	263.12, **C15 H20 O4**	Vinylogous acid	Hulupinic acid	194.05, 126.00, 51.00	126.00, 123.00, 67.02	99.24, -15.28, 1	-	CONE
36	16.75	369.13, **C21 H22 O6**	Prenylflavonoid	Molport-039-338-285 (Prenylflavanone B)	119.05, 193.09, 164.98, 163.00, 124.02, 130.97	119.05, 164.98, 163.00	90.8, -51, 1	-	CO_2R
37	16.75	339.12, **C20 H20 O5**	Prenylflavonoid	Desmethylxanthohumol	119.05, 219.07, 133.06, 65.00	119.05, 133.06, 65.00	99.96, -80.90, 4	-	CO_2R
38	17.01	369.13, **C21 H22 O6**	Prenylflavonoid	5,3′,4′-Trihydroxy-7-Methoxy-8-C-Prenylflavanone (Prenylflavanone A)	135.04, 233.08, 119.05	135.04, 119.05	93, -46, 1	-	CO_2R
39	18.62	407.19, **C25 H28 O9**	Prenylflavonoid	Diprenylflavonone A	317.18, 119.05, 153.00	119.05, 152.05,	96.20, -53.29, 1	-	CO_2R

Abundant fragment ions are from 10, 20 and 40 eV and are listed in descending order of abundance. Precursor ions omitted.
Fragments proportional to voltage included fragments where abundance increased across 10-40 eV.

LEAF-Southern. Aroma hop leaf extract. **CO_2R**-CO_2 extract residue (cv. Herkules). **CONE**-Hop cone (cv. Calypso). **Yakima blend**-Blend of Yakima cultivated hop leaf and cone extracts.

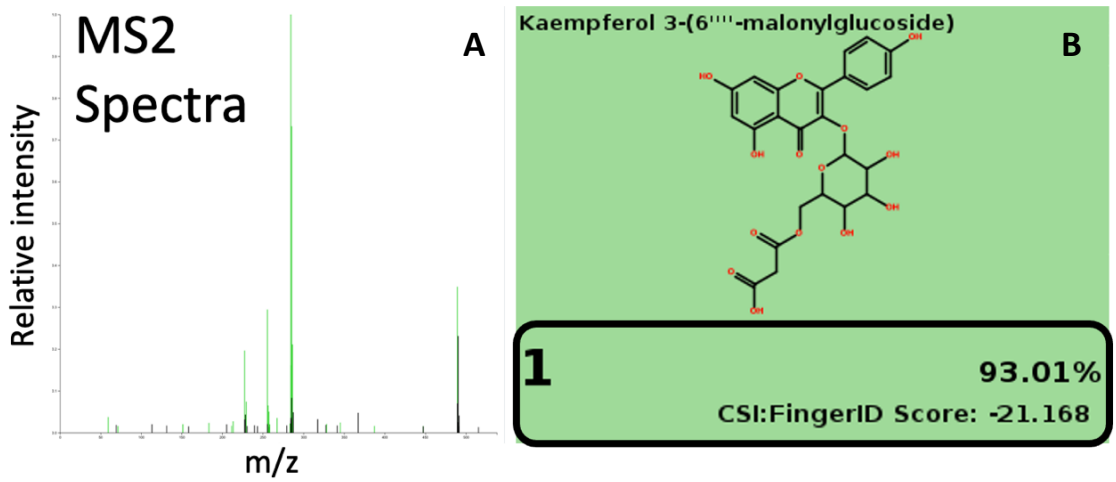


Figure 2.4: MS2 fragmentation patterns for kaempferol 3-O-(6''-malonyl-glucoside) (feature 29) at collision energies 10, 20 and 40 V presented in overlay mode (A). Corresponding CSI:FingerID and rank position for corresponding MS2 (B).

MS/MS fragments were consistent between phenolic subgroups and to published literature. For example, Hu et al. (2024) proposed MS2 fragmentation pathways for quercetin 3-O-rutinoside which are outlined in Figure 2.5. The fragment at *m/z* 301.03 was characteristic of quercetin glycosides (Table 2.4) representing the aglycone quercetin produced by voltage induced cleavage of the glycosidic bond. Hu et al. (2024) proposed that the fragments *m/z* 255.03 and *m/z* 271.03 were attributed to loss of CO_2 and CO respectively which were characteristic of quercetin glycosides (Table 2.4).

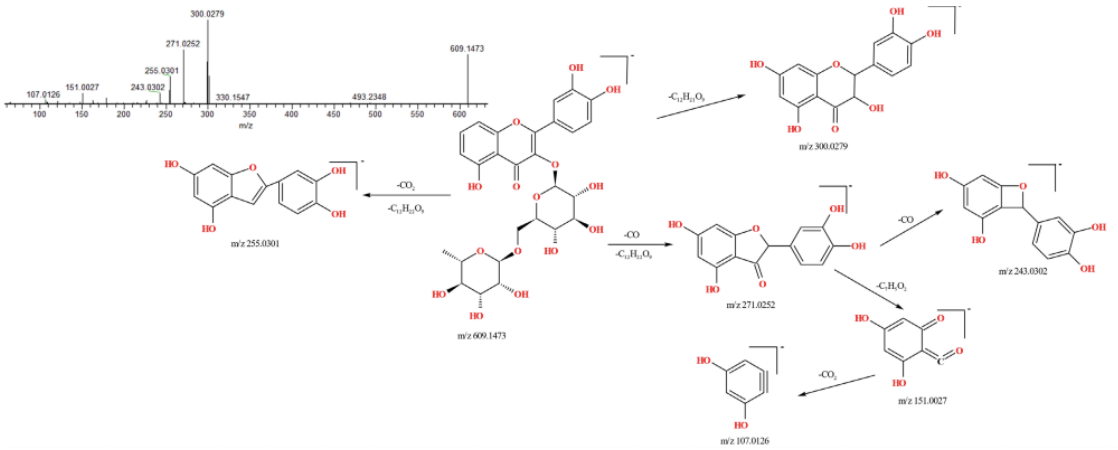


Figure 2.5: Proposed quercetin 3-O-rutinoside MS2 and fragmentation pathways (Hu et al., 2024).

Authentic reference standards were sourced to confirm the structure of features 1, 19, 23, 25, 27 and 28 due to their high abundance in the representative extracts and good SIRIUS and CSI match scores. These were confirmed by comparing RT and mass spectra. Abundance for features 3, 21, 29 and 37 was also very high in at least one of the representative extracts, however standards were not available at the time of analysis.

Poor SIRIUS and CSIFingerID match scores were obtained for a high abundance peak particularly prominent in CO_2R-HERK extract at retention time-11.7 min which can be observed in Figure 2.3 (CO_2R-HERK extract TIC). This peak had an *m/z* of 357.12 consistent with that of co-multifidol glucoside, a phloroglucinol derivative found in high levels in Herkules hop cones (Schmidt and Biendl, 2023b). The EIC at *m/z* of 357.12 from CO_2R-HERK is presented in Figure 2.6. Feature 34 (Table 2.4) had the same m/z (357.12) and was annotated as a phloroglucinol glycoside based on high SIRIUS and CSI:FingerID match scores (Table 2.4).

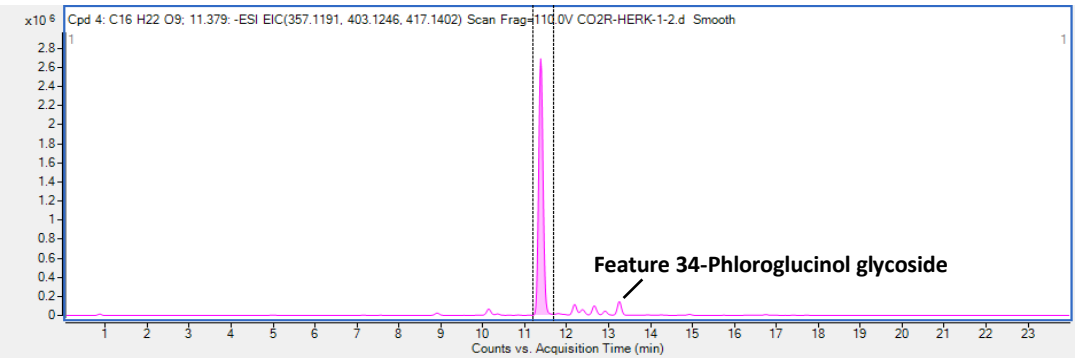


Figure 2.6: Extracted ion chromatogram at m/z 357.12 from CO_2R-HERK extract.

79 phenolic and resin compounds were identified in the representative extracts using authentic reference standards (40) and *in silico* fragmentation patterns (39). Identified compounds were classified based on their structure into the following sub-groups: flavonols and glycosides (20), chlorogenic acids (15), catechins and procyanidins (15), prenylflavonoids (8), phenolic acids and others (21).

Flavonols were made up of the aglycones quercetin and kaempferol and their malonated mono, di and tri glycoside derivatives. Standards were used to identify quercetin (Q), kaempferol (K), quercetin 3-O-glucoside (Q-GLUC), quercetin 3-O-rutinoside (Q-RUT), quercetin 3-O-(6''-malonyl-glucoside) (Q-MG), Quercetin 3-O-sophoroside (Q-SOP) kaempferol 3-O-glucoside (K-GLUC), kaempferol 3-O-galactoside (K-GALAC) and kaempferol 3-O-rutinoside (K-RUT). CSI finger position 1 for feature 19 was K-RUT however this was ruled out with an authentic standard and therefore CSI finger position 2 was used and annotated as kaempferol 3-O-neohesperidoside. Feature 18 was annotated as clitorin (CSI finger position 1), a kaempferol triglycoside found at high concentrations in *C. papaya* leaf extracts (Mohd Abd Razak et al., 2021). However high scores were also found for robinin (CSI finger position 2), which has been reported in Brazilian hop leaves (da Silva et al., 2021). Kaempferol 3-O-(6''-malonyl-glucoside) (feature 29) was particularly abundant in the leaf representative extracts, and has previously been identified in wild hop leaves by McCallum et al. (2019).

Chlorogenic acids were made up of coumaroylquinic acids, feruloylquinic acids and caffeoylquinic acids. Standards were used to identify chlorogenic acid (5-CQA), neochlorogenic acid (3-CQA), 5-O-feruloylquinic acid (5-FQA) and 4,5-dicaffeoylquinic acid (4,5-DCQA). 5 coumaroylquinic acids were identified (features 3-7) and annotated as coumaroylquinic acid isomers (COQA A, B, G, E and D) as high SIRIUS and CSI scores were obtained for a variety of isomers. This was also the case for feruloylquinic acids (features 8-10) and caffeoylquinic acids (features 1, 2) which were annotated as feruloylquinic acid (FQA B, G, D) and caffeoylquinic acid (CQA B) isomers. Phenolic acid standards identified caffeic, coumaric, ferulic, protocatechuic, 4-hydroxybenzoic, hydroxycinnamic acid and 2,5 dihydroxybenzoic acid.

For proanthocyanidins and flavanols, standards were used to identify catechin, epicatechin, dimer procyanidins B1 (P-B1), B2 (P-B2), B3 (P-B3) and a trimer procyanidin C1 (P-C1). CSI

finger position 1 for feature 11 and 12 was P-B1 which was ruled out with a standard and therefore they were annotated as B type dimer 1 and 2 (PBD-1, PBD-2). For prenylflavonols, standards were used to identify xanthohumol, isoxanthohumol and 6-PN. Feature 28 position 1 and 2 was 6-PN and 8-PN respectively however these were ruled out with standards, and it was annotated as desmethylxanthohumol (position 4) based on abundance relative to xanthohumol. Two prenylflavonoids with the same molecular weight as xanthohumol B were annotated as prenylflavanone A and B, whilst a diprenylflavonol was annotated as diprenylflavonone A. 8-PN was confirmed with a standard but was not reported on in this study due to co-elution. A representative extracted ion chromatogram (EIC) of the key target compounds in overlay mode is presented in Figure 2.7.

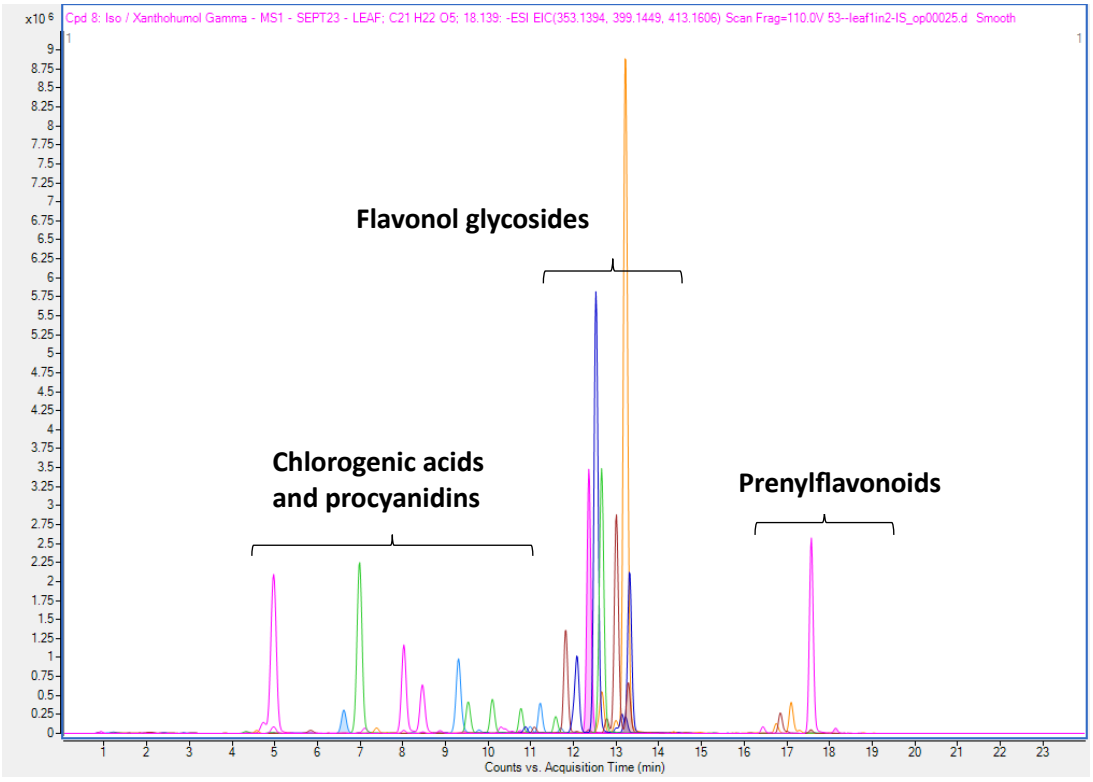


Figure 2.7: Extracted ion chromatogram (EIC) of Yakima leaf and cone representative extract with the main phenolic targets presented in overlay mode.

2.3-Statistical Analysis

Analytical replicates were performed in triplicate and presented as the mean ± standard deviation (SD) of extraction replicates unless otherwise stated. One way ANOVA with Tukey's

HSD post-hoc analysis, t-test analysis, principal component analysis (PCA) and agglomerative hierarchical cluster analysis (AHC) were performed using XLSTAT 3.0. Graphs were produced using Graphpad Prism 10.1.1 and Microsoft Excel (v6.2.14). Heat maps with rows and columns ordered according to AHC (Euclidean distance metric) were produced with Morpheus software (https://software.broadinstitute.org/morpheus).

Chapter 3-Extraction optimisation of phenolics from hop co-products

3.1-Introduction

Over the past few decades, extraction and isolation technology has seen significant advancements, particularly in the recovery of valuable compounds from plant materials. These developments have led to improved extraction efficiency of various groups of compounds including phenolics and essential oils for use in the food, beverage and cosmetic industries (Helmja et al., 2007). Extract composition is largely determined by the choice of solvent and extraction process, and much research has focused on optimizing extraction conditions to target specific compounds (Sanz et al., 2019). Recently this has been applied to optimising extraction of phenolics from agri-food residues using sustainable, 'green' technologies due to their wide availability, natural origins, and antioxidant properties. Among the most widely investigated agri-food residues are pomaces of grapes (Nayak et al., 2018), olives (Alu'datt et al., 2010), and other commonly processed fruits and vegetables.

Hop phenolics derived from agricultural and processing residues have received interest due to their high concentrations and potent antioxidant properties (Proestos and Komaitis, 2009). The most abundant phenolic compounds in hop co-products include prenylflavonoids, proanthocyanidins and flavonol glycosides, although composition depends on variety and type of residue (Jelínek et al., 2014). These compounds have different chemical properties such as polarity and are distributed unevenly between the lupulin glands and the vegetative tissues of hop cones (Stevens et al., 1997). As a result, selection and optimisation of extraction methods is important to maximise extraction of all phenolic components.

The most widely investigated factors for optimising phenolic extraction are pH, extraction solvent, solid: liquid ratio, time and temperature of extraction (Weremfo et al., 2023). Aqueous acetone was the most widely adopted extraction solvent and is generally considered

to be most effective for phenolic extraction from plants, in particular proanthocyanidins (Zam et al., 2012). However, residues of these can be damaging to human health, and costly purification steps are required for their removal. Recently 'green solvents' such as ethanol and CO_2 are widely favoured and are undergoing much development and modification due to their safer use and high solvent power (Marriott, 2010). CO_2 is an effective solvent for hop resins and oils, however it is generally more expensive and less effective for phenolics, depending on the pressure applied (Sanz et al., 2019). Aqueous ethanol is a more suitable solvent for the extraction of hop phenolics, with an ethanol: water ratio around 50: 50 found to be optimal for hop cones (Kowalczyk et al., 2013). However, the optimal ethanol proportion varies depending on the plant material, phenolic composition and other extraction conditions used (Lapornik et al., 2005).

Whilst evaluation of appropriate solvent is important for maximising the extraction of free phenolics, insoluble phenolics can also be present, bound to macromolecules such carbohydrates and cell wall components (Domínguez-Rodríguez et al., 2021). The most common 'green' methods for the release of bound phenolics include pulsed electric field, microwave, ultrasound and enzyme-assisted treatments (Wang et al., 2020). Enzyme assisted extraction has emerged as a preferred treatment due to its mild operating conditions, lower equipment costs and higher selectivity through the tailoring of enzymes used to the plant material (Gligor et al., 2019). Various carbohydrate-hydrolysing enzymes including cellulases, pectinases and xylanses have been studied and shown to improve phenolic extraction from agri-food residues such as grape pomace (de Camargo et al., 2016). These enzymes facilitate phenolic release through cell wall hydrolysis which reduces particle size and liberates bound or intracellular phenolics (Figure 3.1).

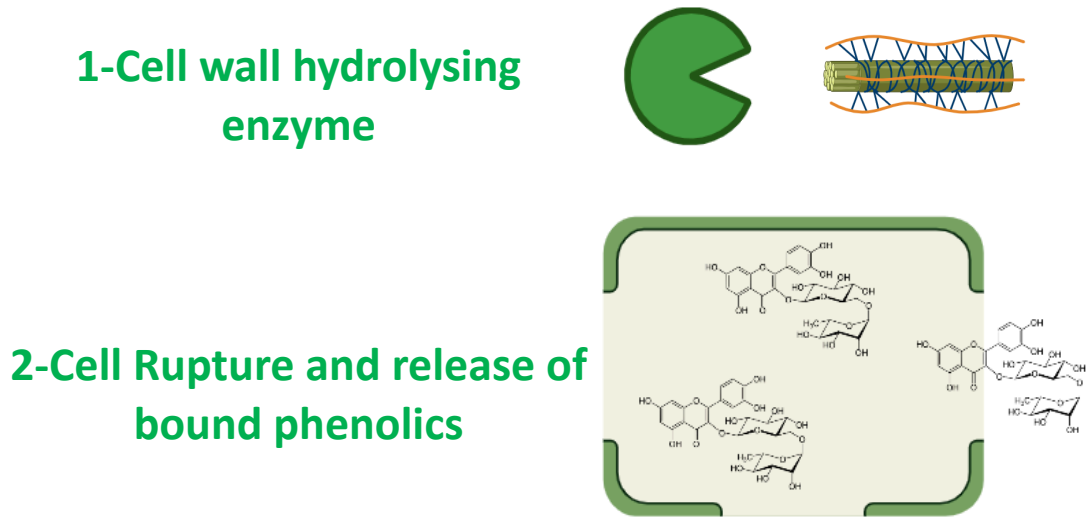


Figure 3.1: Outline of enzyme assisted extraction of phenolics via cell wall breakdown and cell rupture.

This study aimed to optimise the extraction of phenolics from Herkules CO_2 extract residue (CO_2R-HERK) by evaluating the effects of ethanol concentration (30-90%), solid: liquid ratio (100-150 mg/ml) and enzyme assisted extraction. The primary objective was to identify the most effective yet economically viable extraction conditions which could then be applied to a broad range of hop co-products for the characterisation of their phenolic composition and antioxidant properties.

3.2-Materials and Methods

3.2.1-Materials

Hop CO_2 extraction residue from cv. Herkules (CO_2R-HERK) was sourced according to 2.1.2. Commercial enzyme preparation evaluated for phenolic extraction were sourced as detailed in Table 3.1, with optimal conditions based on supplier recommendations.

Table 3.1: Overview of commercial enzyme preparations evaluated for phenolic extraction and their optimal conditions for pH and temperature. Incubation temperature and pH relate to those adopted in this study for each enzyme.

Supplier	Commercial preparation	Dominant enzyme groups	Optimal temperature (°C)	Optimal pH	Incubation temperature (°C)	Incubation pH
DSM (Heerlen, Netherlands)	Enzyme Fis-NF	Cellulases, hemicellulases and β-glucanases	60-90	3-6	60	4.5
	Enzyme Fis-S		60-90	3-6	60	4.5
	Enzyme Max ND	Proteases	40-60	6-8	60	4.5
	DP Max-A		45-65	2-5	60	4.5
	DP Pur-P1	Phospholipase	50-70	3.5-4.5	60	4.5
Novazyme (Bagsværd, Denmark)	Viscozyme	β-glucanases, pectinases, hemicellulases, and xylanases	30-65	4.5-6	40	4.5
IFF (New York City, USA)	Laminex MaxFlow 4G	xylanases and β-glucanases	-	-	40	4.5
Lallemand (Wismar, Germany)	C-MAX	Pectinases	-	-	40	4.5
	EX-V	Pectinases, hemicellulases and cellulases	-	-	40	4.5
	Aromazyme	β-glucosidases	15-65	3.5-6.5	40	4.5

3.2.2-Chemicals

Hydrochloric acid (37%) was sourced from Sigma Aldrich. All other chemicals and reference standards were sourced according to 2.1.1.

3.3-Methods

3.3.1-Extraction of hop phenolics

Hop material (≥100 g) was homogenised and milled with a De'longhi Blade KG49 grinder at speed 12 for 25 s at room temperature until 'fine'. Milled hop material (2 g) was extracted with 20 ml extraction solvent using a Stuart Roller Shaker at 60 rpm for 15 min. The resulting extracts were centrifuged using a Thermo Scientific Heraeus Megafuge 16 Centrifuge for 10 min at 4000 rpm, and subsequently syringe filtered at 0.45 µm prior to analysis.

3.3.2-Enzyme assisted extraction

Between 1-2 g milled hop material was mixed with 9,900-19,800 µL RO water/1 M HCl solution and 100 µL enzyme solution, followed by vortexing for 10 s. Samples were incubated

using a Stuart Roller Shaker at 60 rpm in an incubation chamber for 90-420 min at 30-60 °C based on enzyme supplier recommendations. After incubation, samples were cooled to room temperature for 15 min before the addition of 10,000 µL ethanol followed by extraction at room temperature using a Stuart Roller Shaker at 60 rpm. Samples were then centrifuged for 10 min at 4000 rpm and subsequently syringe filtered at 0.45 µm prior to analysis. Enzyme assisted extractions were performed in duplicate.

3.3.3-Experimental design

A central composite design (CCD) was applied to evaluate the effects of ethanol concentration (30-90%) and solid: liquid ratio (100-150 mg/ml) on response variables: total phenol content (TPC), proanthocyanidin content (PAC), xanthohumol content, alpha acid content and ferric reducing antioxidant power (FRAP). Design-Expert 12 software (Stat-Ease Inc., Minneapolis, USA) was used to generate the design points for samples 1-13 whilst samples 14-17 were added to further explore a specific range of ethanol concentration (42.5-50%). The 17-point experimental design is presented in Table 3.2.

Table 3.2: Experimental runs of CCD to evaluate the effects of ethanol concentration (A) and solid: liquid ratio (B) on phenolic extraction.

Run	A-Ethanol concentration (%)	B-Solid: liquid ratio (mg/ml)
1	81.2132	107.3223
2	60	125
3	30	125
4	60	125
5	38.7868	142.6777
6	60	150
7	60	125
8	60	125
9	81.2132	142.6777
10	60	125
11	38.7868	107.3223
12	60	100
13	90	125
14*	50	100
15*	42.5	100
16*	45	100
17*	47.5	100

Design points added to further explore the ethanol concentration range of 42.5-50% are denoted with an asterisk (*).

3.3.4-Analysis of phenolic content and antioxidant activity

The analysis of phenolic content and antioxidant activity was performed using methodology outlined in 2.2. In brief, extracts were analysed for TPC, PAC and FRAP in triplicate and expressed as caffeic acid (mg CAE/g DM), procyanidin B3 (mg PB3E/g DM) and Trolox equivalents (mg TE/g DM) respectively. Extracts were also analysed for xanthohumol and total alpha acid content in duplicate using LC-DAD using methodology outlined in 2.2.6.1.

3.3-Results and Discussion

3.3.1-Response Surface modelling

To evaluate ethanol % of aqueous extraction solution and solid: liquid ratio, a response surface central composite design (CCD) was used. CCD response surface designs have been extensively used for optimising phenolic extraction as they can be used to investigate interactions between factors at multiple levels from a minimal number of extractions (Liyana-Pathirana and Shahidi, 2005). CO_2R-HERK was selected for phenolic extraction optimisation due to its wide availability with Herkules being the choice to undergo CO_2 extraction. Values for TPC, PAC, xanthohumol content, alpha acid content and FRAP activity for each extraction are provided in Appendix 3, Table 1. Extraction 14-17 were not part of the original design but were added to further investigate xanthohumol extraction within the ethanol concentration range of 42.5-50% after identifying this as the optimal range for TPC, PAC and FRAP extraction. Figure 3.2 presents the response surface contour plots whilst ANOVA p-values and fit statistics for the phenolic variables are presented in Table 3.3.

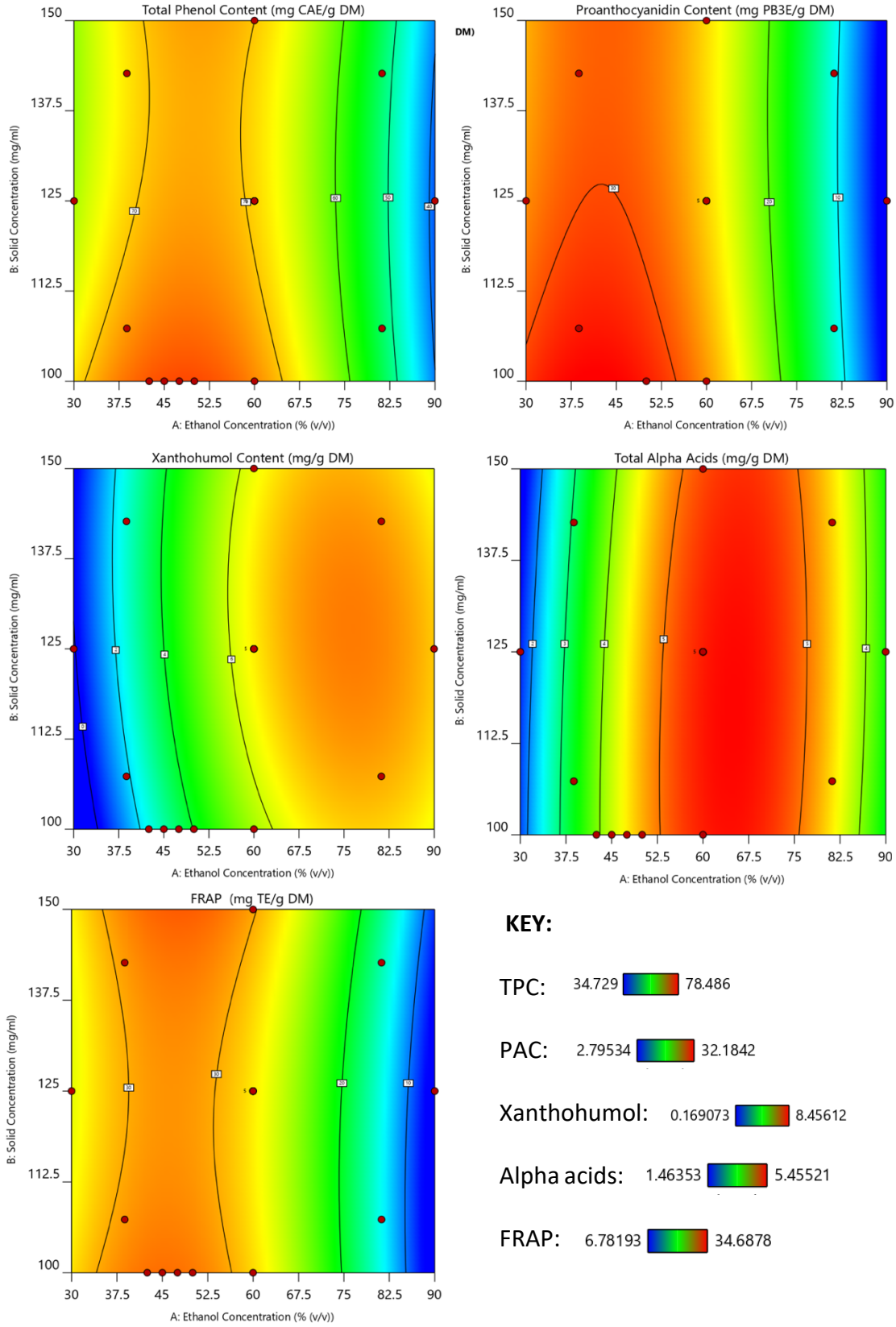


Figure 3.2: Response surface contour plots of total phenol content (TPC), proanthocyanidin content (PAC), xanthohumol content, alpha acid content and FRAP activity as a function of ethanol % and solid: liquid ratio. Values for contour levels are provided in the key in the bottom right corner.

Table 3.3: Statistical analysis of total phenol content (TPC), proanthocyanidin content (PAC), xanthohumol content, alpha acid content and FRAP activity as a function of ethanol concentration, solid: liquid ratio and their interactions.

Source	TPC (mg CAE/g DM)	PAC (mg PB3E/g DM)	Xanthohumol (mg/g DM)	Total alpha acids (mg/g DM)	FRAP (mg TE/g DM)
Model	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**
A-Ethanol concentration (%)	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**
B-Solid: Liquid ratio	0.3195	0.1623	0.3428	0.1162	0.424
AB	0.557	0.6026	0.6092	0.1603	0.5647
A2	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**
B2	0.332	0.4601	0.25	0.2668	0.366
Lack of fit	0.3659	0.0706	0.9979	0.1125	0.1408
			Fit Statistics		
Std. Dev.	2.88	1.41	0.7027	0.1239	1.99
Mean	66.94	23.4	4.86	4.44	26.44
C.V. %	4.3	6.03	14.45	2.79	7.52
R^2	0.9468	0.9852	0.9372	0.9907	0.9536
Adjusted R^2	0.9226	0.976	0.9086	0.9864	0.9326
Predicted R^2	0.7914	0.9147	0.8932	0.9605	0.84
Adeq Precision	21.593	33.1046	17.3281	51.156	22.537

Significant p-values (<0.05) in bold.

TPC: Total phenol content**. PAC**: Proanthocyanidin content.**CAE**: caffeic acid equivalents. **PB3E**: procyanidin B3 equivalents. **Total alpha acids**: Sum of cohumulone and ad/humulone.

The model and the factor A-Ethanol concentration (%) were highly significant ($p < 0.0001$) across all assays, indicating a strong influence on phenolic extraction. In contrast, B-Solid: Liquid ratio was not significant for any of the assays, suggesting it had little or no impact within the analysed range. The quadratic fit for A-Ethanol concentration was highly significant for all assays indicating a non-linear effect which can be seen in Figure 3.2 where an optimal point is reached before becoming less effective.

3.3.1.1-Solid: liquid ratio

The solid: liquid ratio affects both the efficiency and yield of phenolic extraction. Higher ratios can limit extraction efficiency due to reduced solvent penetration and the saturation of phenolic compounds in the solvent (Casazza et al., 2011). Lower ratios generally exhibit better extraction efficiency but are more costly as more solvent is required, which also increases processing and purification costs. In this study solid: liquid ratios in the range of 100-150

mg/ml were evaluated with the aim to maximise phenolic yields whilst minimising extraction costs. Preliminary analysis indicated that ratios above 150 mg/ml significantly decreased the extract fluidity, whilst ratios below 100 mg/ml were not considered economically viable. Although the solid: liquid ratio was not significant for any assay, the extraction efficiency of TPC and PAC declined at ratios above 100 mg/ml (Figure 3.2). Given that proanthocyanidins are reported as the major phenolics in hop cones, a ratio of 100 mg/ml was selected for subsequent trials (Li and Deinzer, 2009). It's important to note that in this experiment 100 mg/ml represented an extreme value (lower boundary) which are less accurately modelled by CCD which represents a limitation of this study. However additional samples (runs 14-17) analysed at 100 mg/ml provide more detail for the ethanol concentration range of 42.5-50%.

3.3.1.2-Ethanol concentration

Aqueous ethanol was selected as the solvent mixture in this study for its lower toxicity compared to conventional extraction solvents, high volatility and well documented effectiveness in hop phenolic extraction (Kowalczyk et al., 2013). In this study we evaluated ethanol concentrations ranging from 30-90% based on preliminary analysis that identified this as the most effective range (data not shown). Phenolic extraction yield is largely dependent on the solvent's polarity, which affects both the composition and concentration of extracted phenolics (Paunović et al., 2015). Pure organic solvents without water typically exhibit poor phenolic extraction, however the addition of water to ethanol increases extraction of phenolics across a range of plant material such as green tea (Xi et al., 2009) and buckwheat (Inglett et al., 2010). It has also been demonstrated by Librán Cuervas-Mons et al. (2013) that very high ethanol concentrations can lead to lower phenolic extraction through cell dehydration thereby limiting phenolic diffusion to the extracting solvent. However, water alone performs poorly for low-polarity phenolic extraction, therefore alcohols which modify polarity are needed.

The optimal ethanol concentration varied depending on the assay, as shown in Figure 3.2. TPC, PAC and FRAP exhibited similar optimal ethanol concentrations of 48.6%, 41.8% and 47.4% respectively, whilst xanthohumol and total alpha acids optimal concentrations were higher at 76.3% and 64.9% respectively. Optimal ethanol concentrations for phenolic and proanthocyanidin extraction of around 40-50% for have been found for a range of plant materials including grape seed meal (Shi et al., 2003), apple pomace (Wang et al., 2018b) and spent coffee ground (Solomakou et al., 2022). The more stable extraction of TPC across the 30-90% ethanol range can be attributed to the range of hop phenolics with different polarities.

Xanthohumol and bitter resins are less polar than other phenolic groups such as proanthocyanidins, and therefore require a higher ethanol proportion for optimal extraction. The drop off in extraction at ethanol concentrations >76.3% for xanthohumol and >64.9% for alpha acids was unexpected as these compounds have exhibited good solubility in organic solvents (de Andrade Silva et al., 2023). This may be due to overfitting of the model or insufficient data points in this concentration range. However, this was not investigated further as extraction of TPC and PAC in this range (65-90% ethanol) was low.

Figure 3.2 shows that the optimal ethanol concentration for TPC, PAC and FRAP activity extraction was within the range of 40-50%. However additional analysis was needed to better understand xanthohumol extraction within this range. Therefore, additional samples were analysed for xanthohumol and total alpha acids at a solid: liquid ratio of 100 mg/ml, for ethanol concentrations 42.5, 45, and 47.5%. As seen in Figure 3.3, xanthohumol and total alpha acid content increased significantly between 42.5-50% with smaller increases between 50-60%. TPC and FRAP also generally increased between 42.5-50% ethanol but decreased between 50-60%. This suggests that 50% ethanol represents a good balance for the extraction

of TPC, PAC, xanthohumol and total alpha acids and it was therefore selected as the optimal concentration to be applied for subsequent extractions.

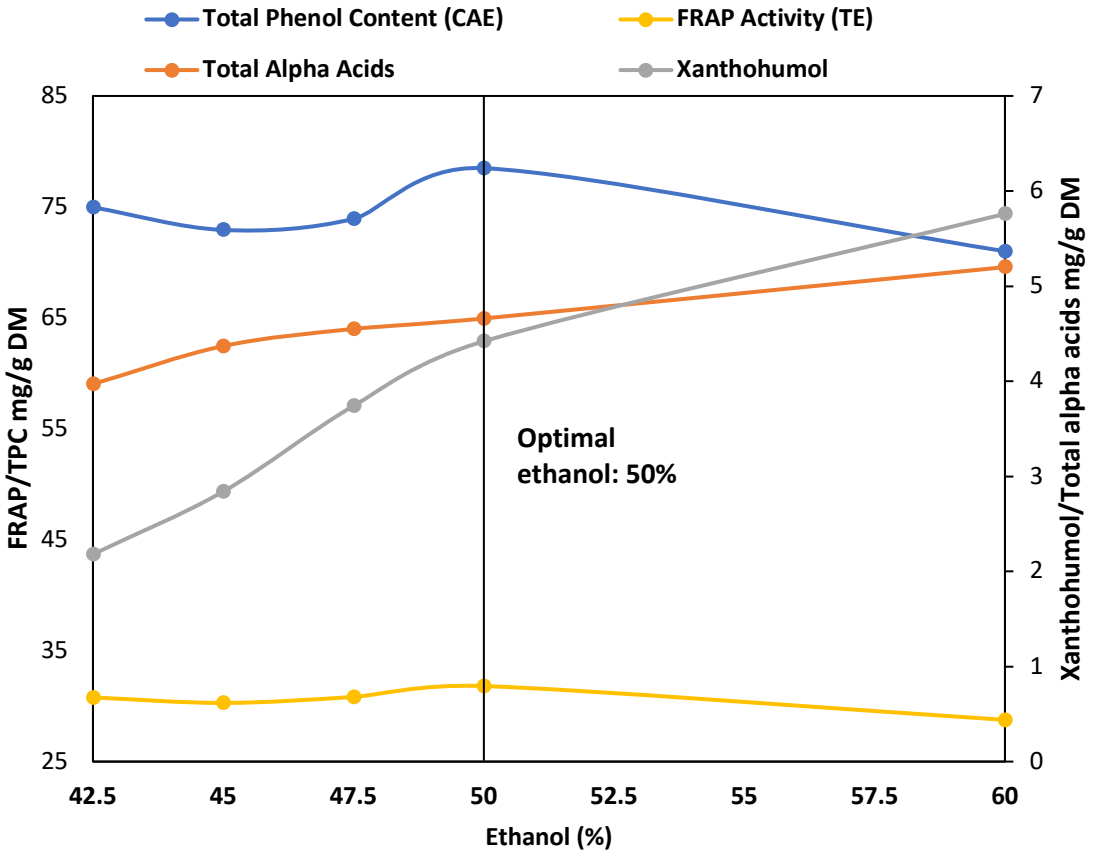


Figure 3.3: Total phenol content (CAE), FRAP activity (TE), xanthohumol and alpha acid content for samples extracted at 100 mg/ml across an ethanol concentration range of 42.5-60%.

Total Alpha Acids-Sum of cohumulone and ad/humulone. **CAE**-Caffeic acid equivalent. **TE**-Trolox equivalent.

3.3.2-Enzyme assisted extraction

Screening trials were conducted to evaluate a range of commercial enzyme preparations suited to phenolic release from plant material using incubation conditions based on supplier recommendations (Table 3.1). The enzymes evaluated included cellulases, hemicellulases, pectinases, proteases as well as combinations of these enzymes, which have shown to enhance phenolic yield from different plant materials (Nadar et al., 2018). To counter the possible buffering action of hop extracts, a second order polynomial was used to model the pH of the aqueous hop extract as a function of 1 M HCl % (v/v), which is provided in Appendix 3, Figure 1.

Ethanol is a known inhibitor of enzymes (Bezerra and Dias, 2005), therefore a two-step extraction method was developed, involving an initial aqueous enzyme incubation step followed by a 50% aqueous ethanol (v/v) phenolic extraction step as outlined in Appendix 3, Figure 2. Manasa et al. (2013) adopted similar conditions for the evaluation of viscozyme assisted extraction of phenolics in ginger (*Z. officinale* Roscoe). Initial evaluations adopted an enzyme incubation solid: liquid ratio of 200 mg/ml so that a final ratio of 100mg/ml was achieved (after ethanol addition) to be consistent with the previous trial evaluating optimal solid: liquid ratio. All enzymes were evaluated at the supplier recommended dose of 1% DM as well as a lower dose of 0.1 % DM. The TPC of EX-V, C-MAX, aromazyme, and Laminex 4G treated and control extracts are presented in Figure 3.4.

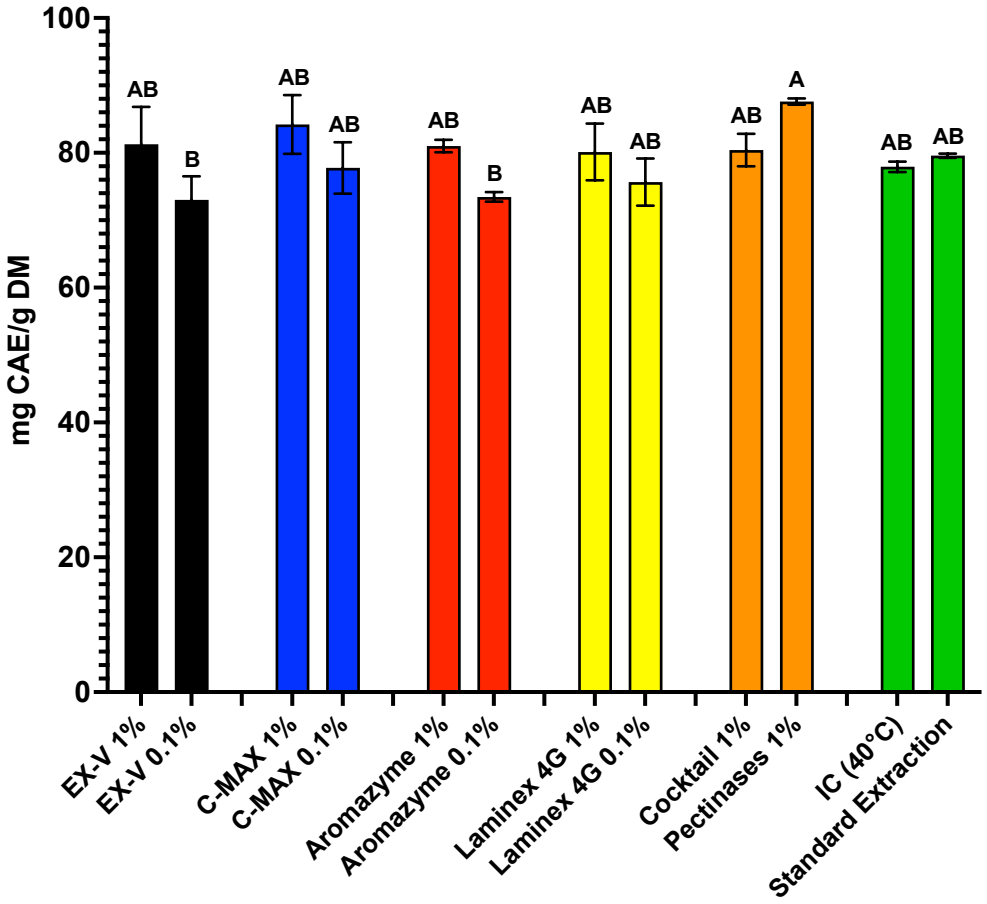


Figure 3.4: Total phenol content (TPC) of enzyme assisted (0.1%, 1%) and control extracts from CO_2R-HERK with enzyme incubations at 200 mg/ml.

EX-V-Pectinases, hemicellulases and cellulases**. C-MAX-**Pectinases**. Aromazyme-**β-glucosidases. **Laminex 4G-**xylanases and β-glucanases. **Pectinases (blend)**-5 mg/ml EX-V, 5 mg/ml C-MAX. **Cocktail (blend)**-2.5 mg/ml EX-V, 2.5 mg/ml-C-MAX, 2.5 mg/ml. **IC**-Incubation control. **Standard extraction**-No incubation.

For all enzyme treated samples, the higher enzyme concentration (1%) resulted in higher TPC compared to the lower concentration (0.1%). However, no significant differences were observed between enzyme treated and control samples. It was hypothesised this might have been due to the high solid: liquid ratio (200 mg/ml) during incubation limiting contact between the enzyme and the plant material. To address this, the second trial evaluating viscozyme and DSM enzyme preparations used an initial solid: liquid ratios of 100mg/ml for a final ratio of 50mg/ml post-ethanol addition. The TPC's of enzyme treated extracts from this trial are presented in Figure 3.5.

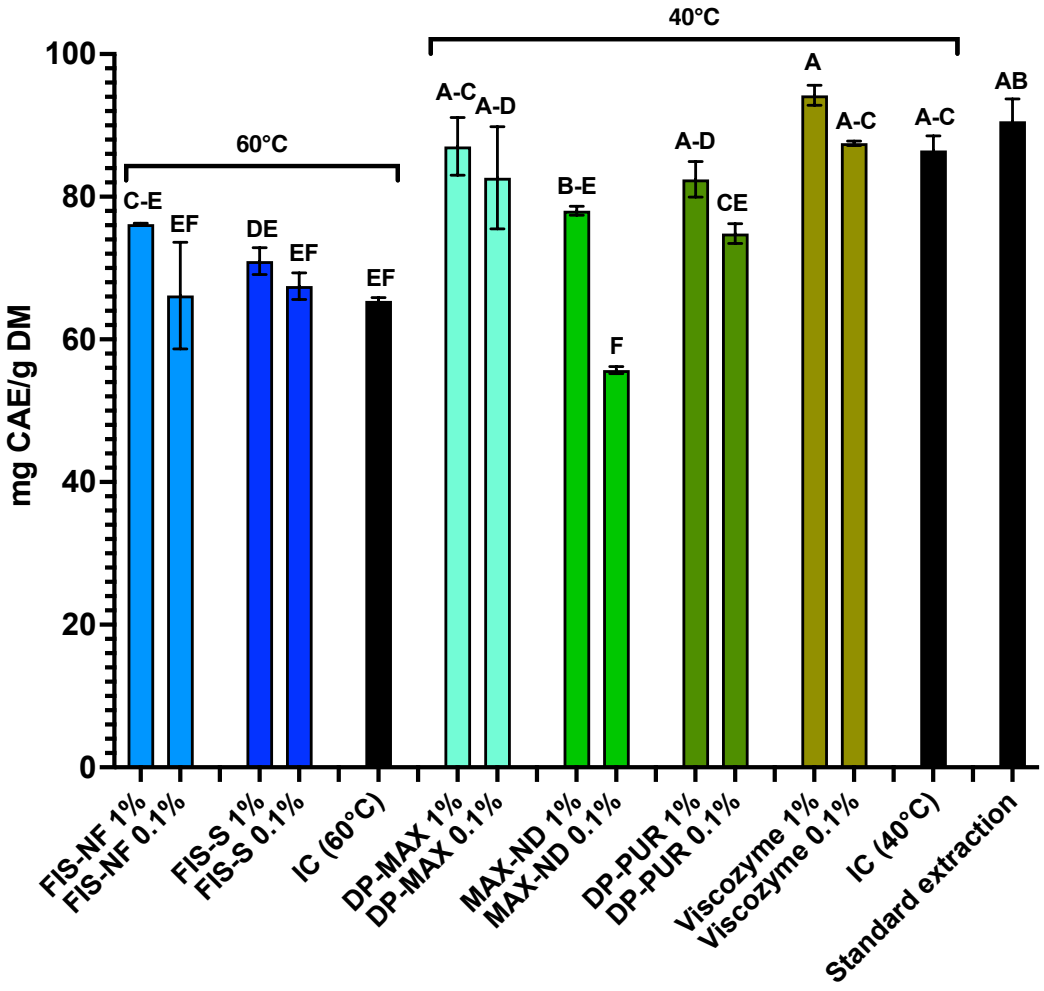


Figure 3.5: Total phenol content (TPC) of enzyme assisted (0.1%, 1%) and control extracts from CO_2R-HERK with enzyme incubations at 100 mg/ml.

FIS-NF/FIS-S-Cellulases, hemicellulases and β-glucanases**. DP-MAX/MAX-ND-**Proteases**. DP-PUR-**Phospholipase**. Viscozyme-**β-glucanases, pectinases, hemicellulases, and xylanases. **IC**-Incubation control. **Standard extraction**-No incubation.

Similar to Figure 3.4, higher enzyme concentrations resulted in higher TPC's, however no extracts were significantly higher than the standard extraction control. All extracts incubated at 60 °C had significantly lower TPC compared to the control, indicating degradation of phenolics at this temperature. Thermal degradation of phenolics from winery waste has been reported at extraction temperatures >60 °C (Lafka et al., 2007). This indicates that enzyme preparations with high optimal temperatures are not suited for the extraction of phenolics from hop co-products. Compared to their incubation controls, enzymes predominantly made up of cellulases such as FIS-NF, FIS-S and Viscozyme were most effective, although differences were small and not significant.

Viscozyme has shown to be particularly effective for enhancing phenolic extraction from various agri-food residues such as unripe apples (Zheng et al., 2009) and the outer leaves of cauliflower (Huynh et al., 2014). The reasons for the much lower enhancement of viscozyme on phenolic extraction in this study is not clear but could be due to shorter incubation times not providing enough time for cell wall degradation necessary to facilitate the release of phenolic compounds. Islam et al. (2023) and Huynh et al. (2014) reported that viscozyme incubation periods >90 min were required for significant increases in phenolic extraction from Cauliflower outer leaves and banana peel extracts. However, it could also be due to a very low content of bound phenolics in CO_2R-HERK, or low enzyme effectiveness at high solid: liquid ratios. For example, a high solid: liquid ratio may limit enzyme activity through reduced enzyme diffusion in the matrix due to high viscosity. However as high solid: liquid ratios are important for the commercial viability of extractions through reduced solvent costs, lower solid: liquid ratios were not investigated in this study.

Overall, enzyme assisted extraction was ineffective across a wide range of commercial enzyme preparations and incubation conditions. Whilst some treatments showed increases in phenolic content relative to the incubation control, these increases were too low to offset

phenolic degradation from incubation, especially at higher temperatures (60 °C). The TPC for the Viscozyme treated extract (1% DM) was higher than the standard extraction, however the increase was modest (<5%) making it unlikely to justify the additional cost of the enzyme preparation and incubation conditions. As a result, 50% aqueous ethanol at 100 mg/ml with no enzyme addition was chosen as the extraction condition for subsequent trials.

Chapter 4-Characterisation of hop co-product phenolic composition and antioxidant activity

4.1-Introduction

In Chapter 3 extraction conditions were optimized to maximize the extraction of phenolics (TPC, PAC, and xanthohumol content) and antioxidant activity (FRAP) extraction from CO_2R-HERK. These extraction conditions were 50% aqueous ethanol (v/v) as the solvent, a solid-to-liquid ratio of 100 mg plant material/ml, without hydrolytic enzyme treatment. The objective of this chapter was to apply these conditions to a range of different hop co-products including T45R, CO_2R and hop leaves to characterise their phenolic composition and antioxidant activity. The overall aim of this study was to identify the hop co-product best suited for purification trials based on phenolic content antioxidant activity, which is detailed in Chapter 5.

4.2-Materials

All materials evaluated in this study were sourced according to section 2.1.2. This included hop leaves and processing residues, hop products and non-hop comparison materials. Processing residues included those from hop cone CO_2 extraction (CO_2R) and T45 pelleting (T45R).

4.3-Methods

4.3.1-Extraction of phenolics from plant materials

Hop materials were extracted using 50% aqueous ethanol (v/v) (E50) using methodology outlined in 2.2.1. Extractions were performed in triplicate per material.

4.3.2-Determination of phenolic content using spectrophotometric assays

Phenolic content of extracts was performed using total phenol content (TPC) and proanthocyanidin content (PAC) assays following methodology outlined in 2.2.4 with results

expressed as caffeic acid (CAE) and procyanidin B3 (PB3E) equivalents respectively. Extracts were analysed in triplicate at 4 mg/ml and 100 mg/ml respectively for TPC and PAC.

4.3.3-Antioxidant analysis of extracts

The antioxidant activity of extracts was analysed using the DPPH, FRAP and ORAC assays and the resulting data was expressed as Trolox equivalents (mg TE/g DM) as detailed in 2.2.5.

For DPPH radical scavenging activity (RSA) extracts were analysed at six different concentrations between 10-350 µg/ml which were used to calculate IC50 values. The RSA of extracts analysed at different concentrations is provided in Appendix 4, Figure 1. RSA of CB-1 and CB-2 extracts exhibited poor linearity above 30%, therefore IC20 values were calculated. For FRAP analysis, extracts were diluted in E50 and analysed at 2 mg/ml in triplicate. For ORAC analysis extracts were diluted in phosphate buffer solution pH 7.4 (PBS) and analysed at 0.05 mg/ml in triplicate.

4.3.4-Chromatographic analysis of extracts

4.3.4.1- Analysis of xanthohumol and bitter acids (HPLC-UV-DAD)

HPLC-DAD was used for the quantitation of xanthohumol, α and β-acids in extracts of hop processing residues and pellets and α and β-acids in leaves following methodology outlined in 2.2.6.1. Extracts were analysed at 100 mg/ml in triplicate in randomised order and data was expressed as w/w DM.

4.3.4.2- Screening and quantitative analysis of phenolics (LC-ESI-qTOF-MS/MS)

For phenolic screening and quantitation LC-ESI-qTOF-MS/MS was used. 4-methyl catechol was used as an internal standard (IS) at a final concentration of 2.5 µg/ml. Extracts were syringe filtered at 0.22 µm, diluted with E50 and analysed at 9.09 mg/ml. Standards were dissolved in E50, diluted across 4 orders of magnitude to 16 concentrations (concentrations ranges provided in Appendix 4, Table 1) and analysed in triplicate at the beginning, middle

and end of the run. Hop extracts were analysed in duplicate per extraction (six replicates per condition). For phenolic screening, peak areas for each compound were normalised to IS and DM thereafter. Fine quantitation for selected polyphenols was performed using standard curves outlined in Appendix 4, Table 1 and expressed as w/w DM. Relative quantitation for phenolic screening was performed by normalising peak areas to IS and DM thereafter.

4.4-Results and Discussion

This study analysed Hallertau Saaz T45R and T45P from the same hop lot and processing batch to determine phenolic transfer during pelleting. Additionally, T45R-SAAZ-CR and T45R-TRAD were analysed to investigate the variability of the phenolic content and antioxidant activity of T45R across different varieties and growing regions. The hop CO_2R samples analysed included two commercially significant varieties, Herkules and Hallertau Blanc, whilst the hop leaf samples consisted of South African varieties, Southern Aroma and Southern Passion. To benchmark the phenolic content of hop co-products, comparison non-hop materials renowned for their phenolic content were also analysed. Whilst comparisons can be made to published literature, the phenolic contents of extracts depends on the extraction condition used, therefore simultaneous evaluation of these materials is recommended (Henrion et al., 2018). Seabuckthorn pomace, a co-product from the juicing of seabuckthorn berries, is of interest due to its bioactive properties (Nour et al., 2021), whilst cranberries, blackcurrants, cinnamon (Rao and Gan, 2014), and tea (Bramati et al., 2003) are renowned for their proanthocyanidin and flavonol glycoside contents.

4.4.1-Phenolic content of hop co-products

Table 4.1 presents the phenolic and proanthocyanidin content of hop co-products, hop products and comparison materials expressed as caffeic acid (CAE) and procyanidin B3 equivalents (PB3E).

Table 4.1: Total phenol (TPC) and proanthocyanidin content (PAC) of hop and comparison materials.

Sample	TPC (mg CAE/g DM)	PAC (mg PB3E/g DM)	PAC %
CO_2R-HB	105.21 ± 1.59 bc	41.46 ± 0.08 cd	39.40
CO_2R-HERK	84.19 ± 4.08 ef	27.27 ± 1.05 h	32.39
T45R-TRAD	86.33 ± 3.22 e	35.29 ± 0.38 fg	40.88
T45R-SAAZ-CR	98.55 ± 0.88 cd	37.4 ± 1.47 ef	37.95
T45R-SAAZ-HT	90.38 ± 2.23 de	49.73 ± 1.39 a	55.02
T45P-SAAZ-HT	84.74 ± 1.7 ef	47.34 ± 1.12 ab	55.86
T90P-SAAZ	138.24 ± 2.62 a	43.97 ± 1.3 bc	31.80
LEAF-SA	106.99 ± 0.48 b	33.61 ± 2.53 g	31.41
LEAF-SP	71.07 ± 2.39 g	19.09 ± 1.05 i	26.86
SBT-POM	91.59 ± 5.84 de	14.54 ± 1 j	15.87
Cinnamon	135.37 ± 2.51 a	39.7 ± 1.19 de	29.32
Tick Tock Tea	91.16 ± 0.56 de	16.37 ± 1.26 ij	17.96
Cranberry-1	47.58 ± 0.98 i	12.71 ± 0.1 jk	26.70
Cranberry-2	77.49 ± 2.17 fg	13.42 ± 0.52 j	17.32
Blackcurrant	56.06 ± 3.62 h	9.49 ± 1.9 k	16.93

Data represent the mean ± standard deviation of triplicate extractions (n=3). Letters represent ANOVA post-hoc groupings.

CAE-caffeic acid equivalent, **PB3E**-procyanidin B3 equivalent, **DM**-dry material. **PAC%**-PAC/TPC *100.

The TPC of hop co-products was similar to those for hop products and was generally higher than comparison materials other than CIN. There was little difference in TPC between co-product types (averages: CO_2R-91.9, T45R-94.4, LEAF-89.7mg CAE/g DM) with differences mainly related to variety. The two hop leaf varieties had the lowest and highest TPC of all co-products analysed even though leaves reportedly contain 3-30 times lower TPC compared to their respective cone (Abram et al., 2015). Although respective S. Passion and S. Aroma cones were not available for analysis, comparisons between the phenolic content of hop cones and leaves are made in chapter 6, for three hop varieties grown in the Pacific Northwest of America.

PAC positively correlated to TPC (R^2 0.65) but was generally higher in hop materials, which also had a higher PAC proportion (PAC %) compared to non-hop materials. Differences in the PAC of hop materials seemed to be related to variety, with Saaz hop materials being

particularly high, especially those from the Hallertau region. Along with variety, the growing environment can impact proanthocyanidin content of hop cones and consequently the processing residue (Li and Deinzer, 2006). Although cranberries are renowned for their A-type proanthocyanidins (Krueger et al., 2013), they, along with cranberry samples and seabuckthorn pomace had the lowest PAC using the acid butanol technique.

T45R has been marketed to brewers as a polyphenol enriched pellet. However, there are conflicting reports regarding the transfer of phenolics between the T45P and T45R. Whilst the TPC of T45P-HT was significantly lower than for T45R-HT there was no significant difference for PAC (Table 4.1). This suggests that proanthocyanidins transfer equally between the two materials, whilst overall phenolic compounds are slightly enriched in the T45R-HT. Branowski and Rolno-Spozywczego (2007) found that half of the polyphenol fraction passed into T45P (using HPLC mass fraction) leaving a relatively rich T45R. Jelínek et al. (2014) found almost double the TPC in Saaz T45R, possibly a result of a lower relative weight of the T45R. However Kowalczyk et al. (2013) found most phenolics are transferred to the T45P for cultivars Magnum and Marynka resulting in low T45R TPC. These conflicting reports may be related to cone and processing variability such as temperature, with low temperatures (-30 °C) necessary to maintain lupulin structure which prevents phenolic oxidative degradation.

4.4.2-Antioxidant activity of hop co-products

The antioxidant activities of hop and comparison extracts are presented in Table 4.2.

Table 4.2: DPPH, FRAP and ORAC antioxidant activities of hop and comparison materials.

Sample	FRAP (mg TE/g DM)	DPPH IC50 (mg TE/g DM)	ORAC (mg TE/g DM)
CO_2R-HB	60.41 ± 0.77 c-e	59.31 ± 6.29 bc	349.4 ± 45.08 ab
CO_2R-HERK	42.31 ± 1.63 h	39.82 ± 3.41 de	292.64 ± 10.39 b-e
T45R-TRAD	44.65 ± 0.45 gh	46.71 ± 1.29 cd	220.58 ± 14.51 fg
T45R-SAAZ-CR	56.02 ± 3.17 c-e	53.23 ± 0.76 b-d	242.16 ± 6.9 d-g
T45R-SAAZ-HT	54.17 ± 0.55 d-f	48.44 ± 0.91 cd	280.44 ± 21.64 c-f
T45P-SAAZ-HT	62.35 ± 1.54 bc	66.44 ± 2.34 b	362.48 ± 4.29 a
T90P-SAAZ	67.65 ± 1.05 bc	64.39 ± 1.71 b	313.17 ± 25.92 a-c
LEAF-SA	60.63 ± 1.86 cd	64.53 ± 2.50 b	297.22 ± 29.08 b-d
LEAF-SP	46.78 ± 1.98 gh	45.52 ± 2.00 c-e	232.41 ± 6.36 e-g
SBT-POM	53.88 ± 0.63 ef	58.60 ± 4.61 bc	76.58 ± 19.41 h
Cinnamon	94.08 ± 6.66 a	141.53 ± 16.40 a	240.2 ± 33.02 d-g
Tick Tock Tea	49.11 ± 1.12 fg	41.86 ± 0.61 de	211.92 ± 13.41 g
Cranberry-1	24.91 ± 0.64 i	19.95 ± 1.16 f	108.31 ± 8.25 h
Cranberry-2	23.57 ± 1.11 i	20.63 ± 0.79 f	118.69 ± 8.71 h
Blackcurrant	43.66 ± 0.89 gh	31.45 ± 1.54 ef	77.14 ± 10.05 h

Data represent the mean ± standard deviation of triplicate extractions (n=3). Letters represent ANOVA post-hoc groupings.

TE-Trolox equivalent, **DM**-dry material.

The antioxidant activity of extracts generally mirrored trends seen in TPC (Table 4.1) with hop pellets (T45P-HT, T90P) and cinnamon extracts exhibiting the highest activities, and berry samples the lowest. The FRAP and DPPH antioxidant activity of hop co-products was comparable to non-hop materials, whereas ORAC activity was generally higher for hop co-products. T45R-HT antioxidant activity was significantly lower than T45P-HT for all assays, in contrast to phenolic content (Table 4.1). PCA was conducted on antioxidant activity and phenolic content variables to investigate differences in antioxidant mechanism across extracts. The PCA biplot for PC1 and PC2, presented in Figure 4.1 explains 91.84% of the variation in the dataset (PC1-73.73%, PC2-18.11%). Correlation analysis between the antioxidant and phenolic assays used in this study is presented in Table 4.3.

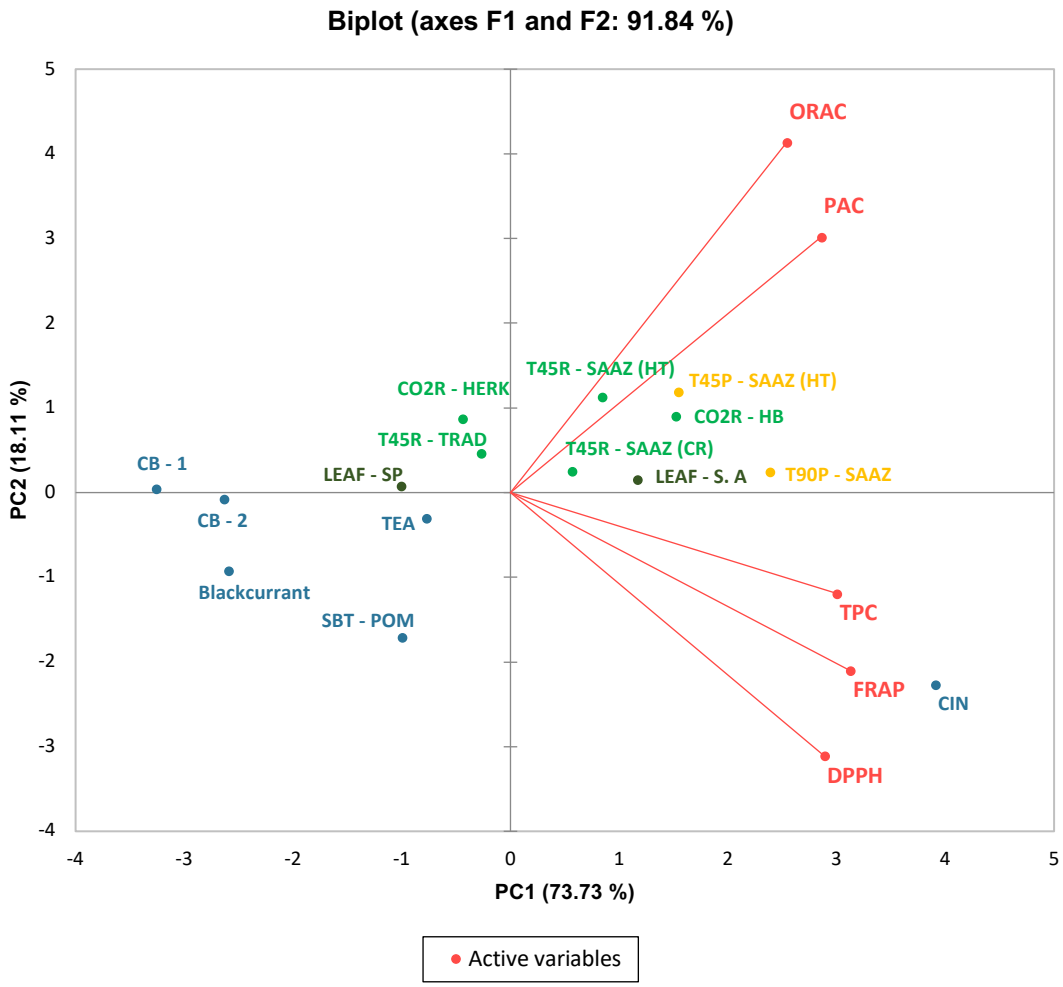


Figure 4.1: PCA plot of materials based on their phenolic content and antioxidant activity. Coloured according to material for hop leaves-dark green, hop processing residues-green, hop pellets-yellow and comparison materials-blue.

Table 4.3: Pearson correlation coefficients for phenolic contents and antioxidant activity.

	TPC (CAE)	PAC (PB3E)	DPPH IC50 (TE)	FRAP (TE)	ORAC (TE)
TPC (CAE)	-	**-**	**-**	**-**	**-**
PAC (PB3E)	**0.65**	-	**-**	**-**	**-**
DPPH IC50 (TE)	**0.77**	**0.53**	-	**-**	-
FRAP (TE)	**0.83**	**0.65**	**0.94**	-	**-**
ORAC (TE)	**0.55**	**0.85**	0.38	**0.52**	-

Correlations where P ≤0.05 in bold font.

The observations plot illustrates the grouping of hop materials, particularly processing residues which score more positively along PC2 due to high PAC and ORAC activities which correlate highly (0.85) (Table 4.3). Hop materials are mainly situated in the top right quadrant whilst LEAF-SP, T45R-TRAD and CO_2R-HERK are positioned in the top left quadrant due to their

lower phenolic content and antioxidant activity. Comparison materials generally had negative PC2 scores due to lower PAC and ORAC activity with CIN positioned in the bottom right quadrant with the highest FRAP, DPPH and TPC values. This indicates hop extracts are more potent peroxyl radical scavengers via hydrogen atom transfer (HAT) than reducers via single electron transfer (SET) possibly due to a significantly higher proanthocyanidin content. Significant high correlations were observed between FRAP, DPPH and TPC (Table 4.3).

4.4.3-Screening and quantitative analysis of phenolics and bitter resins

To identify the most abundant phenolics and resins in hop co-products, quantitation and relative quantitation was performed using LC-MS and LC-DAD analysis. Phenolic variables reported on in this study were those identified in the representative extracts; Cone-CAL, CO2R-HERK and LEAF-SA, as outlined in Table 2.4. Semi-quantitation was performed where an appropriate structurally similar standard was present. Epicatechin gallate, vanillin, 3,4-dicaffeoylquinic acid, trans-3-hydroxycinnamic acid were < LOQ for all materials. Figure 4.2 presents the total concentrations of each phenolic sub-group for each sample whilst Figure 4.3 presents a normalised heat map of all phenolics and resins, with samples and phenolic variables ordered according to AHC. Heat maps are an effective tool for visualising relative differences and identifying groupings between different materials. However, they do not provide information on absolute abundance, and should therefore be interpreted alongside quantitative data for a more comprehensive understanding.

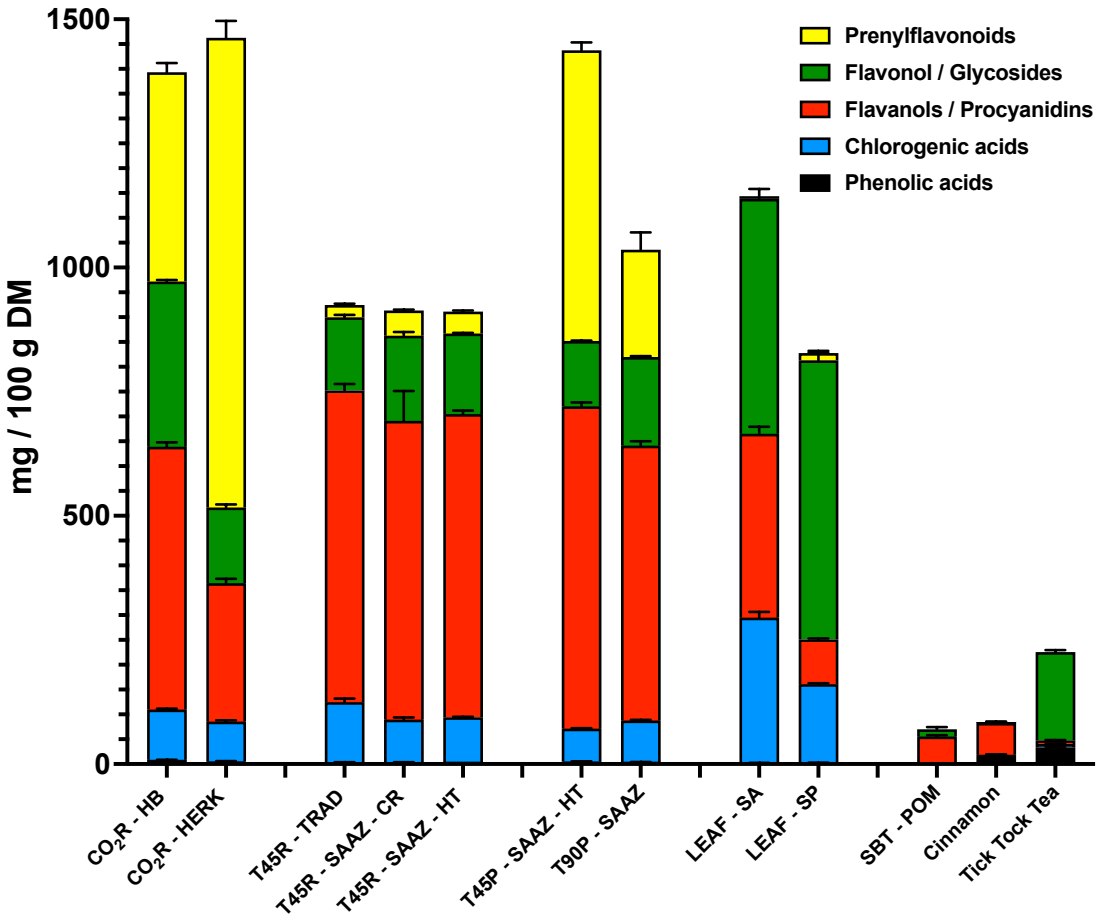


Figure 4.2: Total phenolic content of those quantified using LC-MS and LC-DAD for samples comprising flavonols and glycosides, flavanols and procyanidins, chlorogenic and phenolic acids and prenylflavonoids. Quantitative data of the individual compounds included are presented in Table 4.4.

Data represent the mean ± standard deviation of triplicate extractions (n=3).

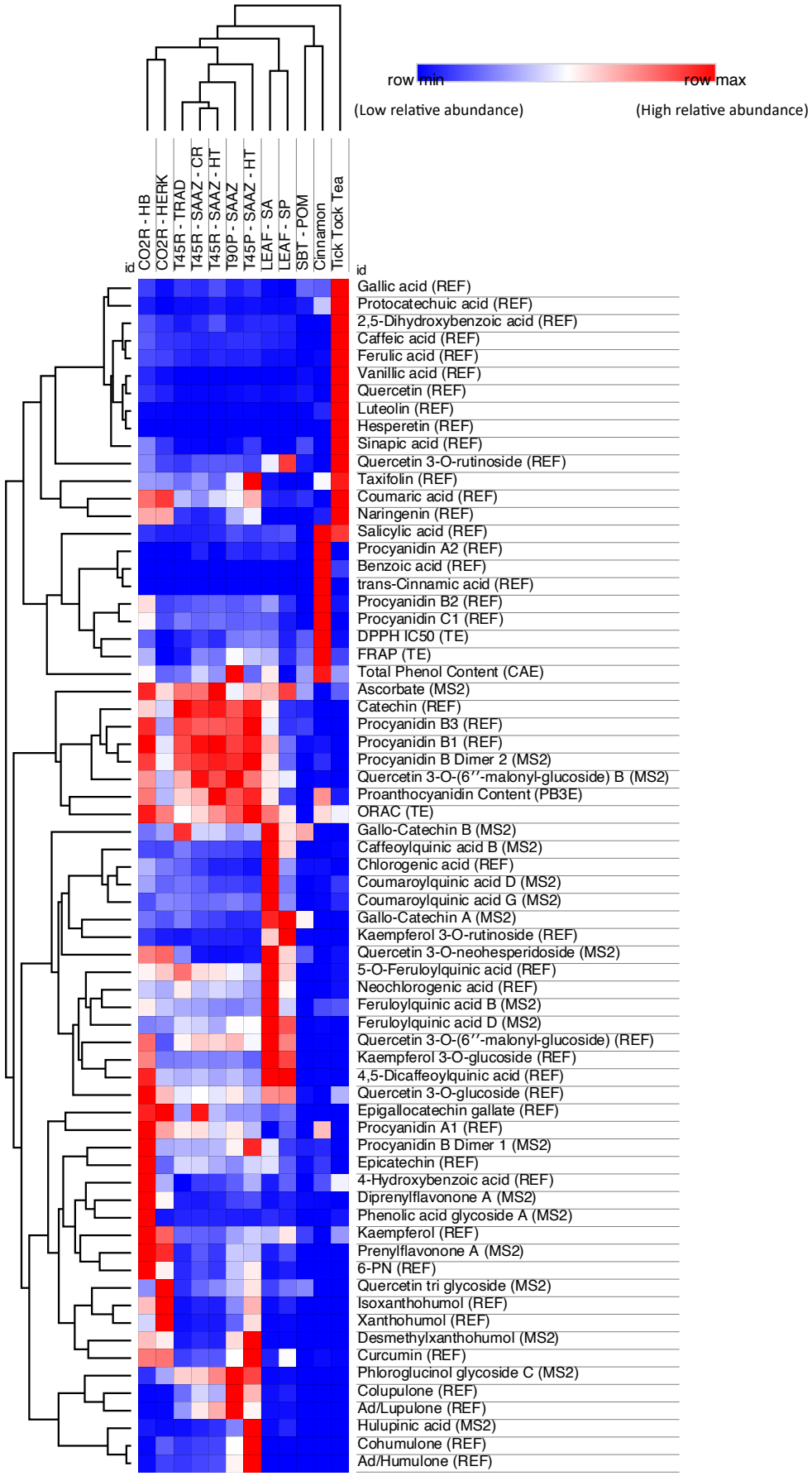


Figure 4.3: Normalised heat map of phenolics and resins for hop and comparison materials. Variables are ordered according to AHC analysis (Euclidean distance).

The most notable difference in phenolic profile was between all hop and comparison materials as shown by the cluster analysis in Figure 4.3. Total phenolics quantified were significantly lower for comparison materials than in hop materials. This contrasts with the TPC data using Folin-Ciocalteu's reagent (Table 4.1) where SBT-POM, TEA and CIN were comparable to hop materials. This difference could be due to the influence of phenolic structure such as the Bors criteria and number of OH groups on the outcome of the Folin-Ciocalteu assay (Platzer et al., 2021). For example, xanthohumol one of the most abundant phenolics in hops interacts poorly with Folin-Ciocalteu's reagent (Gorjanović et al., 2013), possibly leading to an underestimation of phenolic content in these materials. Additionally, the standards used for phenolic identification and quantitation were sourced based on high abundance in hops, and features for MS2 annotation were selected from hop extracts. This limitation might result in underestimating the phenolic content of comparison materials. Nevertheless, most of the phenolics reported in this study such as B-type procyanidins and flavonol glycosides are ubiquitous across different plant materials. Total phenolics of those quantified were significantly lower than those determined by TPC which could indicate the presence of high molecular weight phenolics such as oligomeric proanthocyanidins not detected with the LC-MS method using an m/z range of 50-1700.

Comparison materials were more variable in phenolic composition and generally consisted of flavanols, procyanidins and flavonol glycosides (Figure 4.2). Hop materials generally clustered according to material type with some varietal differences also observed (Figure 4.3). CO_2R was distinguished by higher prenylflavonoid contents, T45R by flavanols and procyanidin content and leaf samples by high levels of flavonol glycosides and chlorogenic acids (Figure 4.3). The greatest varietal differences were observed for CO_2R and hop leaves, with T45R samples being remarkably similar across variety and cultivation location. However, varieties were not consistent between the different co-products analysed which limits varietal

comparisons between co-product types. Table 4.4 and Figure 4.4 present the contents of individual phenolic compounds and bitter acids respectively.

Table 4.4: Concentration of individual phenolics in hop and comparison materials expressed as mg/100 g DM.

	CO_2R-HB	CO_2R-HERK	T45R-TRAD	T45R-CR	T45R-HT	T45P-HT	T90P-SAAZ	LEAF-SA	LEAF-SP	SBT-POM	CIN	TEA
Phenolic and chlorogenic acids												
Gallic acid	0.28 ± 0.04	0.12 ± 0.01	0.25 ± 0.03	0.21 ± 0.01	0.33 ± 0.02	0.25 ± 0.01	0.19 ± 0.00	0.10 ± 0.01	0.08 ± 0.02	0.41 ± 0.01	0.37 ± 0.01	1.68 ± 0.08
Protocatechuic acid	0.65 ± 0.03	0.20 ± 0.01	0.41 ± 0.01	0.42 ± 0.01	0.70 ± 0.01	0.49 ± 0.03	0.39 ± 0.01	0.33 ± 0.02	0.34 ± 0.01	0.65 ± 0.01	3.63 ± 0.05	9.04 ± 0.14
4-Hydroxybenzoic acid	2.57 ± 0.03	1.15 ± 0.07	0.41 ± 0.16	0.65 ± 0.06	0.67 ± 0.01	1.15 ± 0.02	0.81 ± 0.02	0.52 ± 0.03	0.82 ± 0.00	0.41 ± 0.01	0.75 ± 0.06	1.43 ± 0.05
Benzoic acid	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	13.40 ± 0.71	1.58 ± 0.47
Vanillic acid	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	1.31 ± 0.07
Coumaric acid	1.82 ± 0.05	2.02 ± 0.07	1.01 ± 0.04	0.84 ± 0.08	1.12 ± 0.02	1.56 ± 0.05	1.23 ± 0.02	0.44 ± 0.04	0.40 ± 0.05	0.48 ± 0.02	0.29 ± 0.03	2.22 ± 0.04
Caffeic acid	1.13 ± 0.07	0.68 ± 0.04	0.62 ± 0.04	0.46 ± 0.05	0.50 ± 0.01	0.60 ± 0.06	0.60 ± 0.06	0.45 ± 0.01	0.35 ± 0.06	< LOQ	< LOQ	6.44 ± 0.11
Ferulic acid	1.49 ± 0.01	1.34 ± 0.08	0.96 ± 0.02	0.77 ± 0.05	0.94 ± 0.01	0.88 ± 0.02	0.83 ± 0.02	0.53 ± 0.06	0.52 ± 0.05	0.31 ± 0.00	0.44 ± 0.01	8.38 ± 0.07
Sinapic acid	0.49 ± 0.02	0.20 ± 0.06	< LOQ	< LOQ	< LOQ	0.22 ± 0.08	< LOQ	< LOQ	< LOQ	0.28 ± 0.04	< LOQ	1.88 ± 0.11
Chlorogenic acid	7.64 ± 0.08	5.04 ± 0.46	4.09 ± 0.19	2.31 ± 0.35	0.81 ± 0.07	0.12 ± 0.08	0.95 ± 0.09	22.98 ± 0.58	6.31 ± 0.25	< LOQ	< LOQ	< LOQ
Neochlorogenic acid (CQAE)	68.18 ± 1.21	58.14 ± 1.61	92.00 ± 6.37	65.12 ± 3.46	72.61 ± 0.65	54.51 ± 0.41	66.02 ± 0.76	171.66 ± 7.04	88.80 ± 0.55	< LOQ	< LOQ	5.12 ± 0.30
CQA B (CQAE)	11.91 ± 0.27	11.25 ± 0.55	20.01 ± 0.59	13.17 ± 0.52	11.91 ± 0.13	7.56 ± 0.13	11.32 ± 0.34	83.66 ± 3.33	49.01 ± 0.62	< LOQ	< LOQ	1.14 ± 0.07
4,5-Dicaffeoylquinic acid (3,4-DCQAE)	13.49 ± 0.16	5.41 ± 0.17	4.92 ± 0.21	5.38 ± 0.18	4.63 ± 0.08	4.23 ± 0.06	5.32 ± 0.11	14.37 ± 0.32	14.49 ± 0.28	< LOQ	< LOQ	< LOQ
Flavanols and procyanidins												
Catechin	261.74 ± 0.36	185.15 ± 4.87	438.42 ± 2.84	410.97 ± 51.15	419.62 ± 1.76	419.86 ± 4.65	366.44 ± 2.94	232.44 ± 9.07	46.16 ± 0.66	36.04 ± 1.94	5.77 ± 0.07	5.45 ± 0.11
Epicatechin	47.27 ± 0.31	9.43 ± 0.32	19.81 ± 0.65	19.68 ± 0.86	16.07 ± 0.14	19.66 ± 0.37	12.89 ± 0.06	20.72 ± 0.49	9.00 ± 0.21	0.95 ± 0.05	5.21 ± 0.15	< LOQ
Epigallocatechin gallate	0.83 ± 0.00	0.86 ± 0.00	0.53 ± 0.02	0.84 ± 0.05	0.55 ± 0.02	0.51 ± 0.04	0.52 ± 0.01	0.46 ± 0.02	0.48 ± 0.04	< LOQ	< LOQ	< LOQ
Procyanidin A1	1.02 ± 0.06	0.65 ± 0.06	0.49 ± 0.02	0.52 ± 0.05	0.36 ± 0.02	0.29 ± 0.05	0.49 ± 0.09	< LOQ	< LOQ	< LOQ	0.59 ± 0.16	< LOQ
Procyanidin A2	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	3.02 ± 0.04	< LOQ
Procyanidin B1	59.34 ± 4.92	26.55 ± 1.23	50.97 ± 5.29	57.91 ± 0.98	59.45 ± 2.26	56.85 ± 0.28	52.08 ± 2.53	37.30 ± 2.24	13.19 ± 0.25	< LOQ	2.23 ± 0.06	< LOQ
Procyanidin B2	11.49 ± 0.17	2.61 ± 0.19	3.23 ± 0.24	3.82 ± 0.66	4.07 ± 0.58	4.23 ± 0.29	3.55 ± 0.05	6.17 ± 0.18	1.98 ± 0.02	0.36 ± 0.00	20.27 ± 0.96	0.74 ± 0.03

Procyanidin B3	90.04 ± 0.78	32.33 ± 1.00	85.69 ± 2.36	79.91 ± 4.98	80.55 ± 0.75	97.47 ± 0.97	83.08 ± 1.11	46.04 ± 1.18	11.52 ± 0.12	13.67 ± 0.44	1.64 ± 0.04	1.73 ± 0.10
Procyanidin BD 1 (PB1E)	34.27 ± 0.28	11.54 ± 0.22	12.05 ± 0.42	11.67 ± 0.40	12.36 ± 0.15	31.81 ± 0.98	17.98 ± 0.63	15.48 ± 0.44	3.94 ± 0.16	2.03 ± 0.07	2.64 ± 0.25	< LOQ
Procyanidin BD 2 (PB1E)	12.09 ± 0.11	6.50 ± 0.08	11.46 ± 0.57	12.34 ± 0.52	12.94 ± 0.65	13.67 ± 0.01	12.44 ± 0.18	7.61 ± 0.33	2.72 ± 0.30	< LOQ	1.18 ± 0.09	< LOQ
Procyanidin C1	11.13 ± 1.86	3.15 ± 1.01	5.22 ± 0.40	4.31 ± 0.50	4.78 ± 0.87	4.27 ± 0.50	4.06 ± 1.25	4.04 ± 0.19	1.04 ± 0.06	< LOQ	21.59 ± 0.74	< LOQ
Flavonols and flavonol glycosides												
Kaempferol	1.26 ± 0.09	1.02 ± 0.05	0.25 ± 0.02	0.24 ± 0.01	0.22 ± 0.02	0.51 ± 0.01	0.40 ± 0.04	0.46 ± 0.04	0.69 ± 0.02	0.16 ± 0.02	< LOQ	0.37 ± 0.02
Quercetin	2.91 ± 0.20	1.66 ± 0.14	0.28 ± 0.03	0.24 ± 0.03	0.21 ± 0.02	0.80 ± 0.02	0.49 ± 0.04	0.38 ± 0.03	0.60 ± 0.10	1.20 ± 0.11	< LOQ	27.65 ± 1.09
Taxifolin	0.15 ± 0.02	0.14 ± 0.01	0.11 ± 0.02	0.14 ± 0.00	0.10 ± 0.01	0.49 ± 0.02	0.23 ± 0.02	0.02 ± 0.01	< LOQ	0.07 ± 0.01	0.24 ± 0.03	0.46 ± 0.02
Q-RUT	29.50 ± 0.24	15.06 ± 0.65	11.95 ± 0.32	18.91 ± 0.97	19.49 ± 0.16	15.13 ± 0.06	19.27 ± 0.30	51.27 ± 1.98	98.79 ± 3.29	5.25 ± 0.03	< LOQ	110.39 ± 1.95
Q-GLUC (RE)	105.82 ± 0.61	67.17 ± 3.07	48.55 ± 1.39	53.09 ± 2.17	47.98 ± 0.15	40.34 ± 0.26	57.63 ± 0.39	76.67 ± 2.82	79.15 ± 1.89	2.80 ± 0.06	0.33 ± 0.02	37.14 ± 0.42
Q-NEO (RE)	11.78 ± 0.23	12.64 ± 0.30	4.24 ± 0.18	0.52 ± 0.12	0.31 ± 0.07	0.67 ± 0.02	0.33 ± 0.08	15.80 ± 0.93	9.36 ± 0.43	2.80 ± 0.03	< LOQ	0.46 ± 0.14
Q-MG (RE)	66.49 ± 1.29	13.73 ± 0.58	43.69 ± 1.50	50.74 ± 2.23	49.09 ± 0.42	40.59 ± 0.16	53.15 ± 0.32	83.94 ± 3.04	62.75 ± 0.86	< LOQ	< LOQ	0.22 ± 0.02
K-RUT (RE)	23.13 ± 0.24	11.22 ± 0.38	7.78 ± 0.21	14.62 ± 0.73	13.54 ± 0.05	9.61 ± 0.23	13.22 ± 0.21	121.46 ± 6.67	202.91 ± 5.81	1.54 ± 0.13	0.67 ± 0.12	0.39 ± 0.05
K-GLUC (RE)	91.80 ± 0.14	29.58 ± 1.35	30.54 ± 0.95	33.10 ± 1.16	31.23 ± 0.50	23.87 ± 0.16	34.04 ± 0.37	123.15 ± 4.20	107.83 ± 2.50	0.24 ± 0.04	0.40 ± 0.12	0.52 ± 0.04
Prenylflavonoids												
Xanthohumol	377.54 ± 13.89	909.67 ± 25.65	21.76 ± 2.14	44.31 ± 1.14	39.52 ± 1.56	515.73 ± 11.01	177.47 ± 28.02	4.70 ± 0.10	12.70 ± 0.29	-	-	-
6-PN	2.72 ± 0.03	1.42 ± 0.09	0.18 ± 0.01	0.38 ± 0.01	0.24 ± 0.00	1.44 ± 0.00	1.03 ± 0.01	0.03 ± 0.00	0.10 ± 0.00	-	-	-
Isoxanthohumol	10.11 ± 0.12	16.01 ± 0.95	0.41 ± 0.01	1.06 ± 0.06	0.87 ± 0.01	10.42 ± 0.12	4.35 ± 0.04	0.28 ± 0.03	1.13 ± 0.04	-	-	-
Desmethylxanthohumol (XNE)	36.15 ± 1.75	30.99 ± 2.08	2.40 ± 0.23	4.67 ± 0.29	3.56 ± 0.15	57.61 ± 2.42	32.93 ± 0.87	0.17 ± 0.03	0.38 ± 0.04	-	-	-
Other												
Hesperetin	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	0.17 ± 0.02
Naringenin	0.27 ± 0.02	0.27 ± 0.03	0.04 ± 0.01	0.03 ± 0.01	0.04 ± 0.03	0.19 ± 0.01	0.14 ± 0.00	< LOQ	< LOQ	< LOQ	0.02 ± 0.00	0.41 ± 0.01

Data represent the mean ± standard deviation of triplicate extractions (n=3).

RE: rutin equivalent, **CQAE**: chlorogenic acid equivalent, **3,4-DCQAE**: 3,4-Dicaffeoylquinic acid equivalent, **PB1E**; procyanidin B1 equivalent, **XNE**: xanthohumol equivalent.

4.4.3.1-Bitter Resins and Prenylflavonoids

Xanthohumol, α- and β-acids are unique to hops and are the most abundant secondary metabolites in hop cones (up to 300 mg/g DM) (Patzak et al., 2015). The concentrations of bitter resins and prenylflavonoids across different hop materials are presented in Figure 4.4 and Table 4.4 respectively. The abundance of these compounds appeared to be primarily driven by processing mechanism. Prenylflavonoid concentrations followed the order: CO_2R > T45P and T90P > T45R > leaves. Bitter acids were more variable but generally showed a trend of: T90P and T45P > CO_2R and T45R > leaves.

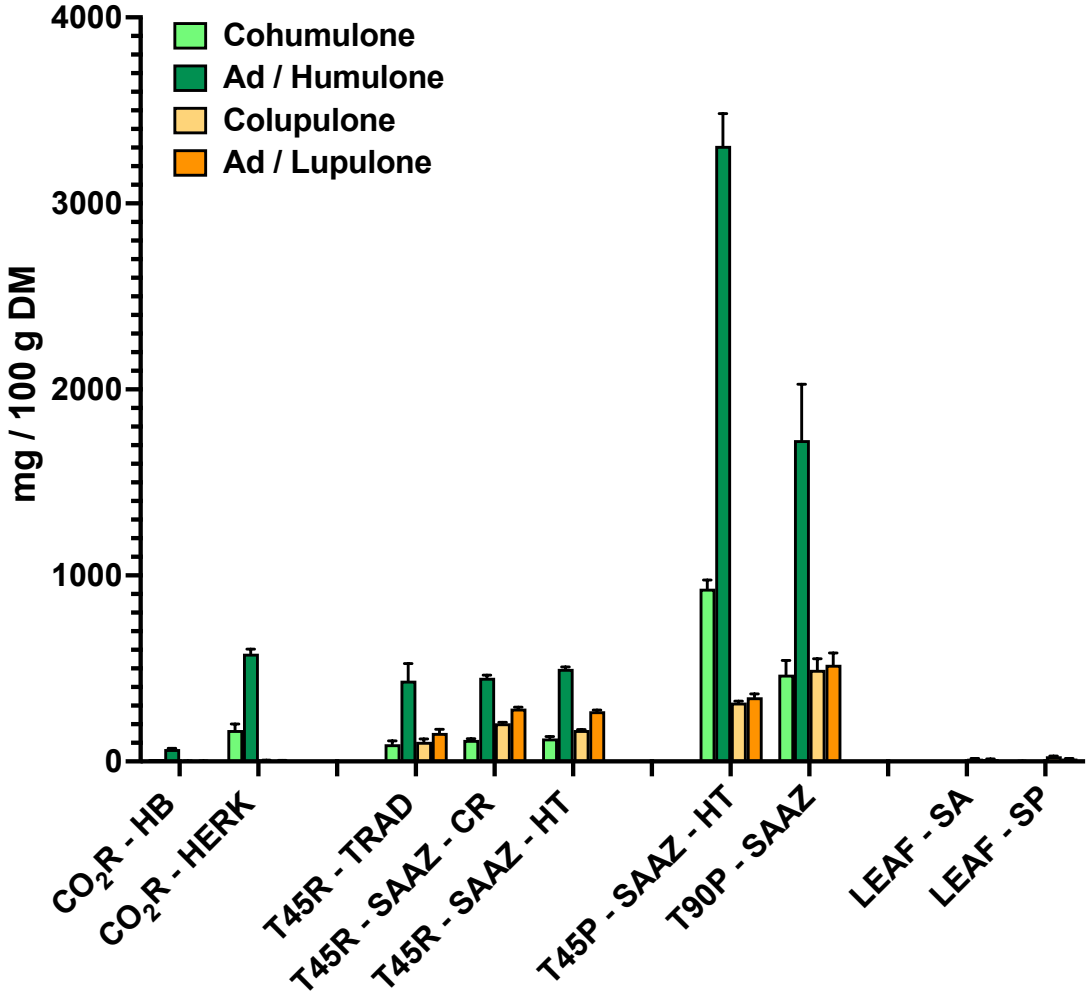


Figure 4.4: Contents of α- and β-acids in hop co-product, product and leaf extracts.

Data represent the mean ± standard deviation of triplicate extractions (n=3).

Xanthohumol was the principle prenylflavonoid for all hop materials with desmethylxanthohumol, 6-PN and isoxanthohumol found in lower concentrations (Table 4.4). CO_2R had the highest xanthohumol concentrations at 377.5 and 909.7 mg/100 g DM for CO_2R-HB and CO_2R-HERK respectively. These were higher than the content of their cones as reported by Hopsteiner (2024) varietal data sheets. This confirms literature reports that the concentration of xanthohumol in CO_2R depends largely on variety but is generally present at a higher concentration than in respective cones (Moens et al., 2020). 6-PN, isoxanthohumol, diprenylflavonone A and prenylflavonone A were also found at higher levels in CO_2R compared to other hop materials, whilst desmethylxanthohumol was present at highest levels in T45P-SAAZ-HT (Table 4.4, Figure 4.4).

T45R materials contained lower levels prenylflavonoids but comparable α-acid levels relative to CO_2R. Herkules and Hallertau blanc hop cones have significantly higher α-acid concentrations compared to Saaz and Tradition as reported by Hopsteiner (2024). This indicates that CO_2 extraction is more effective than T45 pelleting for resin extraction. T45R-SAAZ-HT had significantly lower levels of prenylflavonoid and bitter resins compared to T45P-SAAZ-HT which is in line with Jelínek et al. (2014), who reported a total xanthohumol and bitter resin content of <0.5% w/w in T45R-Saaz. Interestingly the transfer of β-acids to T45P-SAAZ-HT was lower than that of α-acids and prenylflavonoids (Figure 4.4). The reason for this is unclear but could be attributed to higher susceptibility of β-acids in T45P-SAAZ-HT to degradation, as evidenced by the elevated levels of hulupinic acid (an oxidation product of β-acids) in T45P-SAAZ-HT (Figure 4.3) (Burton et al., 1964). As expected, hop leaves contained lower levels of resins and prenylflavonoids. Hop leaf bitter acid and prenylflavonoid content is further explored in Chapter 6.

4.4.3.2-Flavanols and Procyanidins

Flavanols and procyanidins in hop materials were primarily composed of catechins and B-type procyanidins. Catechin was the most abundant, followed by PB3, PB1 and epicatechin, with A-type procyanidins present at much lower levels (Table 4.4). In contrast, CIN had a higher proportion of A-type procyanidins, PB2, and PC1, whilst SBT-POM showed a higher proportion of gallocatechins (Figure 4.3). The ratios between catechin and other flavanols and procyanidins were significantly higher than those reported in the literature. For example catechin: epicatechin ratios in Saaz hop materials ranged from 20.9-28.4 whilst Chenot et al. (2023) reported a ratio of around 3.8-3.9 for Saaz cones.

Flavanols and procyanidins were quantitatively the main components in all T45R and Saaz materials, whilst in hop leaves and CO_2R their levels varied depending on variety (Figure 4.3, Table 4.4). Analysis by Schmidt and Biendl (2023a) reported that Tradition and Saaz varieties contained higher levels of flavanols and procyanidins compared to Herkules and Hallertau Blanc, suggesting that variety may have a greater influence than processing method on flavanol and procyanidin content in residues. The contents of flavanols and procyanidins in T45R-HT was similar to that of T45P-HT which is consistent with the PAC for these materials outlined in Table 4.1. Overall these findings align with literature reports highlighting Saaz as a particularly rich source of proanthocyanidins (Linforth et al., 2015).

4.4.3.3-Flavonols and their glycosides

Flavonols are the amongst the most widespread phenolic compounds in plants and are found either as aglycones or glycosidically bound. In hops the main aglycones are quercetin, kaempferol and myricetin with a diverse range of flavonol glycosides formed through various sugars bound at different positions on the phenolic structure (Dušek et al., 2021). The dominant hop flavonol glycosides identified in hop co-products in this study were quercetin and kaempferol 3-O-glucosides and 3-O-rutinosides with no 7-O- or 4-O-glycosides identified

(Figure 4.3, Table 4.4). Schmidt and Biendl (2023b) similarly identified quercetin and kaempferol 3-O-glucosides and 3-O-rutinosides as the major glycosides of hop cones. Although malonylated glycosides have been tentatively identified in hops (Santagostini et al., 2020), this study confirms their identity with a standard.

All flavonol glycosides were semi-quantified as rutin equivalents (RE) as performed by Stanius et al. (2022). The total flavonol glycoside content of hop materials ranged from 132.00-562.09 mg RE/100 g DM, which was significantly higher than the total aglycones. Higher levels of flavonol glycosylation and malonylation has been reported for a variety of plant materials including hop cones (Shimoda et al., 2007) and shoots (Maietti et al., 2017), however there is limited literature on their prevalence in hop leaves. Their prevalence is likely due to glycosylation enhancing solubility, distribution and metabolism by facilitating transport through membranes (Šamec et al., 2021).

Hop leaves had the highest total flavonol glycoside contents of all materials, in particular kaempferol 3-O-rutinoside. The proportion of kaempferol glycosides relative to total glycosides was also higher in hop leaves (50.9-54.4%) compared to other hop materials (9.3-35.1%). Flavonol glycosides profiles were similar across hop processing residues and pellets whereas non-hop comparison material profiles were significantly different from those of hops. CIN and SBT-POM total glycosides was low at 24.20 and 160.93 mg RE/g DM respectively whilst TEA had similar total flavonol glycoside levels to hop materials and was predominantly composed of quercetin 3-O-glucoside and 3-O-rutinoside. Additionally, TEA also had the highest level of aglycones compared to other materials, primarily composed of quercetin (Table 4.4).

Flavonol glycosides were generally present at higher levels in T45R-HT compared to T45P-HT (Table 5.4). This is in line with work by Goiris et al. (2014) who found that T45P of Hersbrucker Spät had a rutin content of 102 mg/100 g DM compared to respective T90 pellet at 117

mg/100 g indicating glycosides are predominantly transferred to the T45R. Interestingly the aglycones, taxifolin and naringenin, were found in significantly higher levels in the T45P and in amounts corresponding to the degree of lupulin enrichment during processing (T45P > T90P > T45R) (Table 4.4). This could indicate that aglycone flavonols are predominantly located in lupulin whilst glycosides are in the bracts of hop cones.

4.4.3.4-Phenolic and Chlorogenic acids

Phenolic acids, categorized as either benzoic or cinnamic acids based on their precursors, are low molecular weight flavonoid precursors (Natella et al., 1999). Among these, chlorogenic acids, esters of quinic acid and hydroxycinnamic acids like caffeic, ferulic, and p-coumaric acid, are prominent (Lu et al., 2020).

Total phenolic acids concentrations were found to be low in all hop materials (2.51-8.44 mg/100 g DM) with caffeic, ferulic, coumaric and protocatechuic acid being the most abundant. Comparison materials generally exhibited higher but more variable phenolic acid contents (2.55-33.95 mg/100 g DM) (Figure 4.3, Table 4.4). Conversely, hop materials showed a higher abundance of all isomers of feruloylquinic, coumaroylquinic, caffeoylquinic and dicaffeoylquinic acids (Figure 4.3). Total chlorogenic acids for those quantified ranged from 66.6-292.7 mg/100 g DM in hop materials with comparison materials < 10 mg/ 100 g DM. Hop leaves had higher abundance of all chlorogenic acids followed by CO_2R followed by T45R and hop pellets. Neochlorogenic acid (3-O) was the most abundant caffeoylquinic acid in all hop materials which is in agreement with Luzak et al. (2016) who found similar ratios of chlorogenic acids in spent hops with neochlorogenic as the most abundant.

4.4.3.5-Phenolic enrichment

It has previously been shown that chlorogenic acids and flavonol glycosides were disproportionately transferred to T45R-SAAZ-HT whilst CO_2R was a richer source of prenylflavonoids. However, as varieties were not consistent between materials sourced for

this study it is challenging to distinguish between effect of variety and processing mechanism. Schmidt and Biendl (2023a) reported quantitative data for phenolics in German Hallertau Blanc, Herkules, Tradition and Czech Saaz hop cones from the same crop year and country of cultivation as those analysed in this study which can be used to assess degree of enrichment. Ratios between values reported in cones and concentrations reported in this study processing residues (Table 4.4) were calculated and normalised for each compound and presented as a heat map (Figure 4.5). Fold differences for flavonol glycosides and procyanidins were generally higher for CO_2R as compared to T45R. This suggests that, although T45R had a higher overall concentration of procyanidins, this is likely due to the initial concentration in the cones rather than differences in the processing mechanism and that CO_2R is likely a richer source of phenolics depending on variety. For example, Saaz is known to be a rich source of procyanidins whilst Herkules contains lower levels. This is an important distinction if the most prevalent residue available on the market changes in the future from CO_2R-HERK and T45-SAAZ to different varieties.

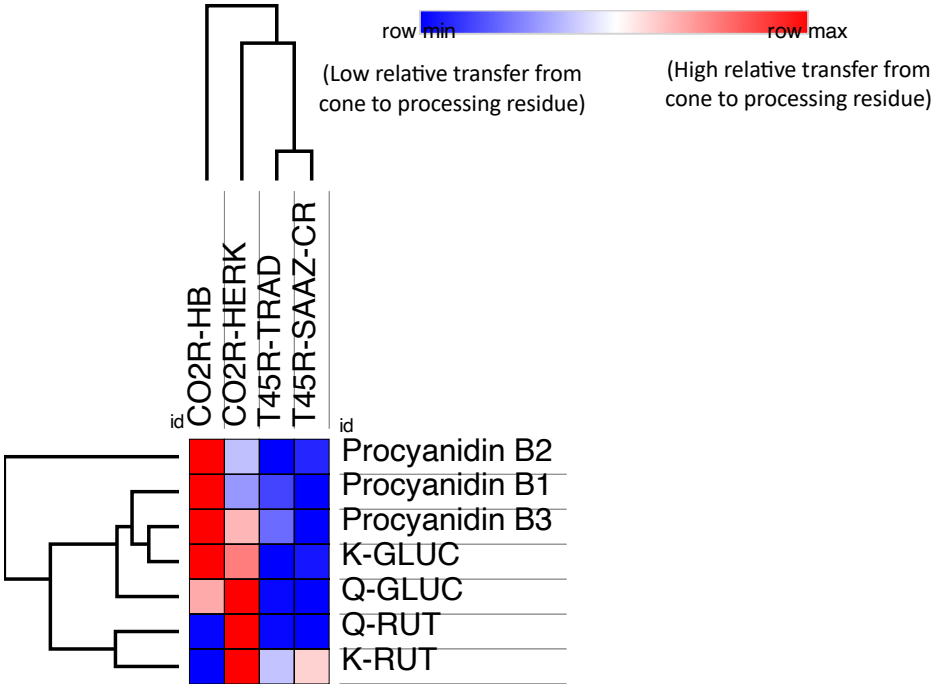


Figure 4.5: Normalised fold differences in phenolic content between hop cones (as reported by Schmidt and Biendl (2023b) and processing residues (CO_2R and T45R) for the same variety, region of cultivation and crop year (Table 4.4).

4.4.4-Multivariate Analysis

Multivariate analysis is particularly useful for identifying trends and patterns in complex datasets and is routinely used to classify materials based on their phenolic profile (Zielinski et al., 2014). Techniques such as PCA reduce data dimensionality which can aid data visualisation, however it can also lead to the loss of minor components that may still be quantitatively significant (Granato et al., 2018). Therefore, it remains important to evaluate the contributions of the individual phenolic variables to the principal components and consider their absolute abundance and bioactive properties.

To investigate trends in phenolic differences between hop materials, PCA and HCA analysis was conducted. Non-hop materials and phenolic variables with IS normalised abundance < 0.05 in the highest sample were omitted from analysis. PCA produced six principal components with eigenvalues > 1 explaining 98.18% of variation. These components were analysed using Wards hierarchical clustering method (Euclidean distance) to measure dissimilarity based on phenolic variables. The resulting dendrogram showing clustering of samples into groups is presented in Figure 4.6.

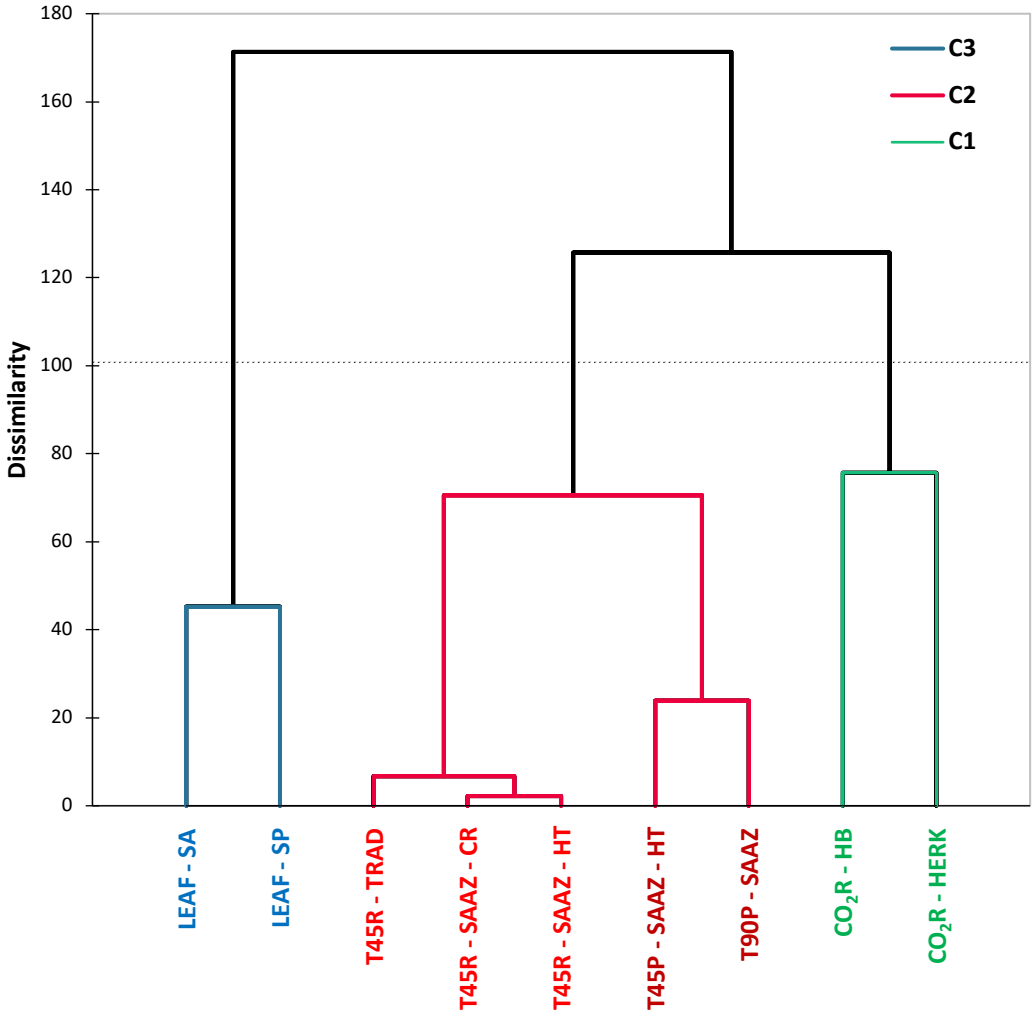


Figure 4.6: Dendrogram of cluster analysis of hop materials based on phenolic profile and antioxidant activity. Samples coloured according to material; Leaves-blue, CO_2R-green, T45R-light red, T45P and T90P-dark red.

Three clusters were identified based on Hartigan index truncation, which separated samples by material type. Cluster 1 (C1, green) contained CO_2R, Cluster 2 (C2, red) included T45R, T45P and T90P, whilst Cluster 3 (C3, blue) contained hop leaves. C3 was the most distinct cluster with C2 and C1 more similar, likely due to both containing cone-derived hop materials. C1 and C3 were more homogenous than C2 which sub-clustered by material with T45R grouped separately from T45P and T90P. The PCA biplot for PC1 and PC2 (Figure 4.7) explains 62.67% of the total variation in the data set (PC1-37.2%, PC2-25.48%). The observations plot highlights groupings based on material type with tighter groupings observed for leaf and T45R

compared to CO_2R. Leaves were distinguished from other materials along PC1 with higher scores whist CO_2R were distinguished from T45R along PC2 with higher scores. T45R was also distinguished from T45P and to a lesser extent T90P with higher PC1 scores although all within the same cluster (C2).

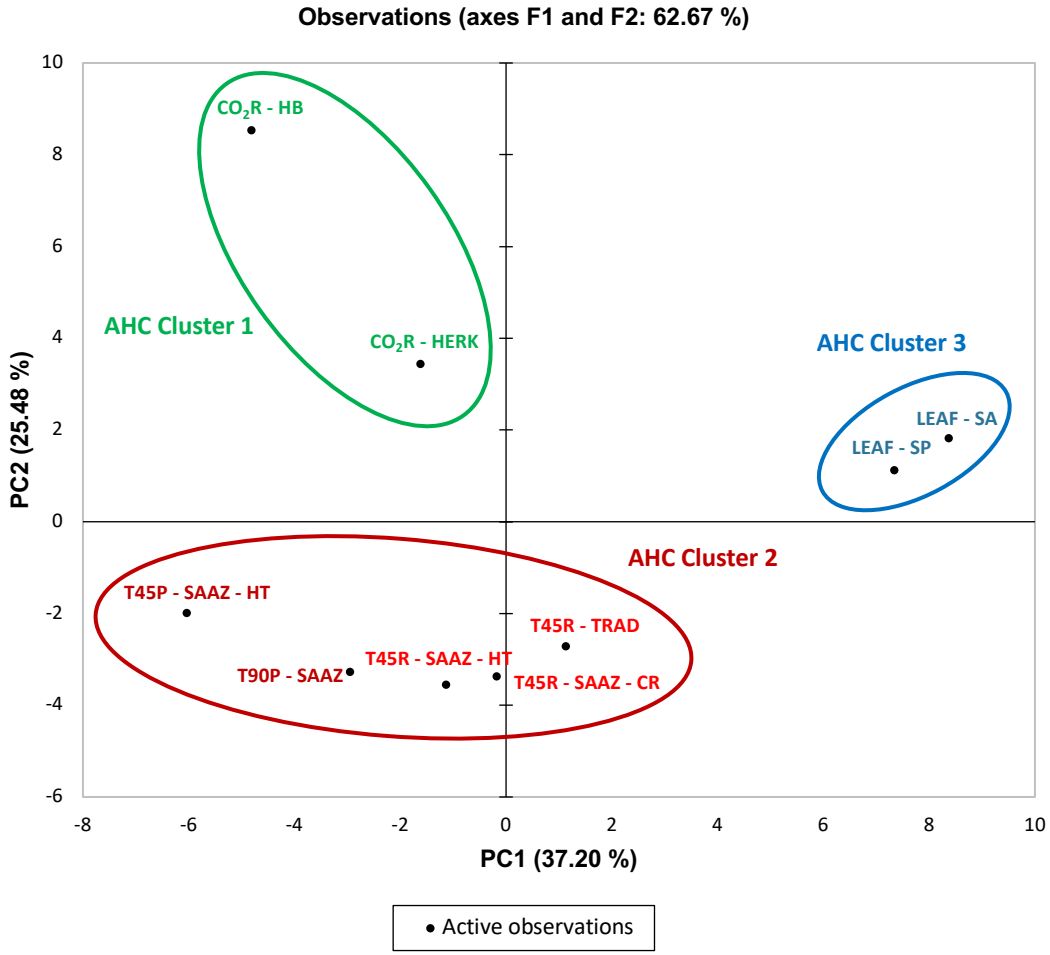


Figure 4.7: PCA plot of materials based on phenolic content and antioxidant activity. Samples coloured according to material; Leaves-blue, CO_2R-green, T45R-light red, T45/T90P-dark red.

To investigate which phenolics and antioxidant mechanisms drive sample groupings, correlations between phenolic variables and principal components were investigated (Appendix 4, Table 3). PC1 showed strong positive correlations to chlorogenic acids

(caffeoylquinic acid B, 0.90; neochlorogenic acid, 0.80; feruloylquinic acid D, 0.83), kaempferol glycosides (K-RUT, 0.83) and gallo-catechins (gallo-catechin A, 0.91) and showed negative correlations to prenylflavonoids (desmethylxanthohumol, -0.80; 6-PN, -0.74), procyanidins (procyanidin B3, -0.73) and phenolic acids (coumaric acid, -0.84). PC2 was positively correlated to quercetin glycosides (Q-GLUC, 0.91; Q-NEO, 0.81) aglycones (kaempferol, 0.94) and negatively correlated to beta acids (Ad/Lupulone, -0.77), catechin (-0.61) and phloroglucinol-glycoside C (-0.76). PC3 (data not shown) was positively correlated to Q-MG (0.79), DPPH (0.73) and FRAP (0.73) antioxidant activity and negatively correlated to Q-TG (-0.73). It therefore seems that the phenolics driving co-product differences are prenylflavonoids for CO_2R, beta-acids, flavanols and procyanidins for T45R and kaempferol glycosides, gallo-catechins and chlorogenic acids for hop leaves.

4.4.5-Correlation between phenolic composition and antioxidant activity

To investigate the contribution of phenolic and resin variables to extract antioxidant activity, correlation analysis was conducted across all hop materials. Correlations are visualised as a heat map (Figure 4.8) with columns and rows ordered according to HCA. Three primary clusters were identified: Cluster 1 (C1) with low correlations to all assays, Cluster 2 (C2) with high correlations to all assays and Cluster 3 (C3) with high correlations to ORAC but lower correlations to other assays. Flavanols and procyanidins had the strongest correlations to antioxidant activity (predominantly C2) whilst flavonol glycosides and chlorogenic acids had the weakest correlations (predominantly C1). Quercetin 3-O-malonyl glycosides were exceptions with both located within C2. Quercetin 3-O-malonyl glucoside has been shown to be a more potent antioxidant compared to its respective mono-glycosides, although not to quercetin (Nowak et al., 2014, Panat et al., 2015). Proanthocyanidins, especially those with a high molecular weight explored as beer flavour (Mikyška et al., 2022) and colloidal stability agents and to improve lautering speed (Karabín et al., 2018). This indicates that T45R could have promise for the development of antioxidant and brewing stabilisation aids.

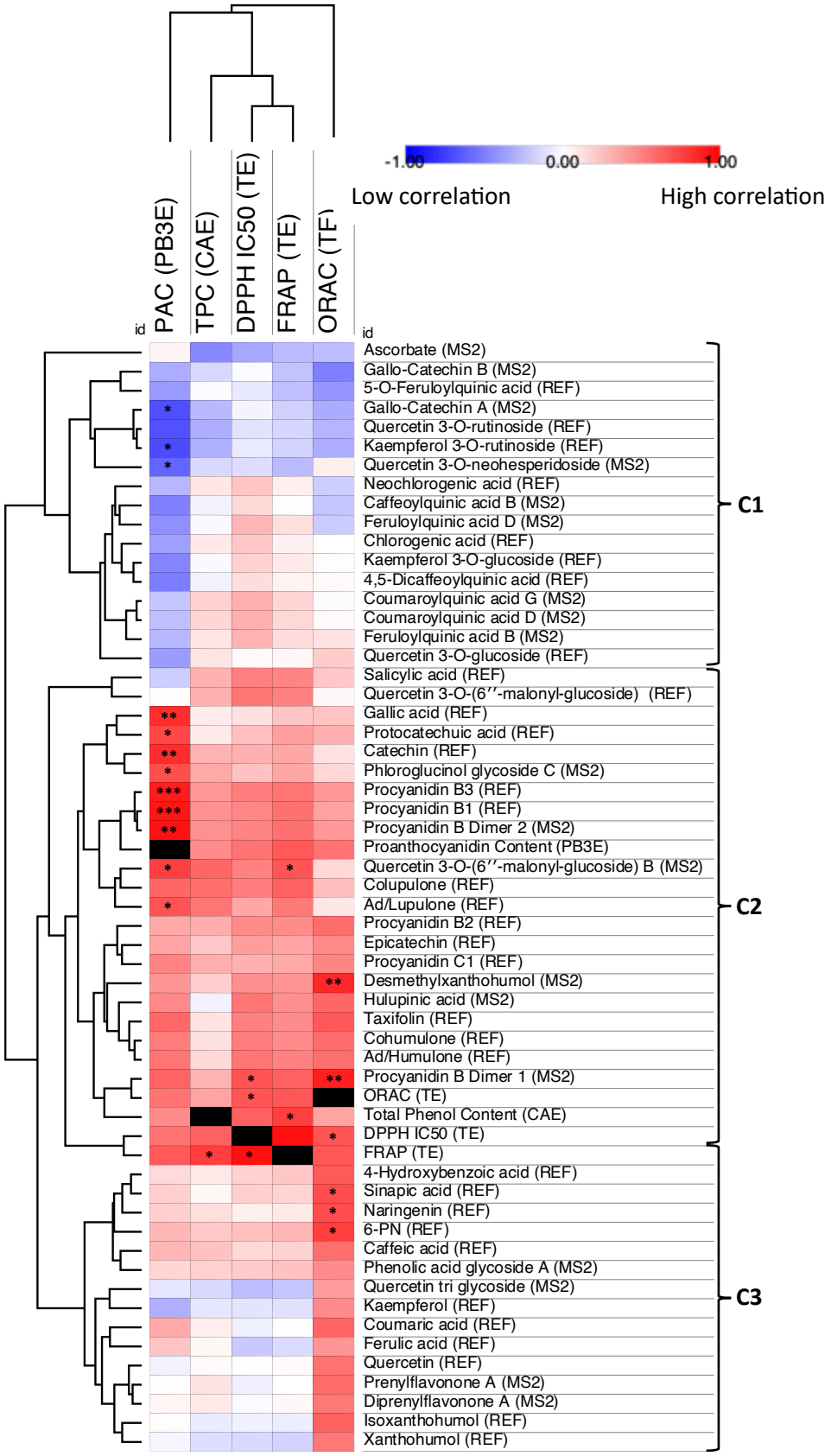


Figure 4.8: Heat map depicting Pearson's correlation coefficients (-1 – 1) between hop material antioxidant activity and phenolic variables with columns and rows ordered by AHC.

Correlation significance levels are denoted as follows: * P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001.

Prenylflavonoids were predominantly found within C3 with high correlations to ORAC values (significant correlations ($P<0.05$) for desmethylxathohumol and 6-PN) which indicates higher peroxyl radical scavenging activity. Prenylflavonoids and bitter resins have been shown to exhibit poor ET based antioxidant activity such as is evaluated using DPPH and FRAP assays (Zhang et al., 2014). However, they are potent hydroxyl and peroxyl radical scavengers (Van Hoyweghen et al., 2010). Xanthohumol and other hop prenylflavonoids have also received much interest in the pharmaceutical and nutraceutical industries due to their anti-cancer, anti-inflammatory, metal chelating and hypoglycaemic effects suggesting disease prevention applications (Abiko et al., 2022, Liu et al., 2015).

As a result of the properties of prenylflavonoids, research has focused on optimising the extraction and purification of xanthohumol from hop material which has resulted in numerous patents which is summarised in a recent review by de Andrade Silva et al. (2023). Biendl (2012) trialled PVPP as an adsorption resin for the purification of xanthohumol from ethanolic hop extracts. He demonstrated selective desorption with ethyl acetate was effective and achieved a prenylflavonoid purity of >90 %. PVPP also exhibits high affinity for other major hop polyphenols such as catechins (Magalhães et al., 2010), hence future trials could evaluate PVPP as an adsorption resin for the purification and fractionation of phenolics from hop co-products.

CO_2R-HERK generally had comparable but lower phenolic contents and antioxidant activity compared to the other hop co-products. However, the high concentration of xanthohumol indicates it could have value as a starting material for the development of prenylflavonoid rich extracts. Considering the high availability of CO_2R-HERK (as it is the choice variety for CO_2 extraction), it seems to be the most suited hop co-product (of those analysed in this study) for the development of purified hop phenolic extracts.

Chapter 5-Evaluation of purification techniques for phenolic compounds from hop co-products

5.1-Introduction

Crude phenolic extracts from plant materials often contain undesirable compounds, including pigments, proteins, carbohydrates, terpenes, and fats. These impurities can reduce the bioactive properties of extracts and impart negative sensory characteristics. For commercial applications, it is important to remove these impurities whilst concentrating desirable phenolic compounds. A variety of purification techniques have been evaluated for this purpose, the most common of which are adsorption and ultrafiltration which are outlined in Figure 5.1 (Kelly et al., 2019).

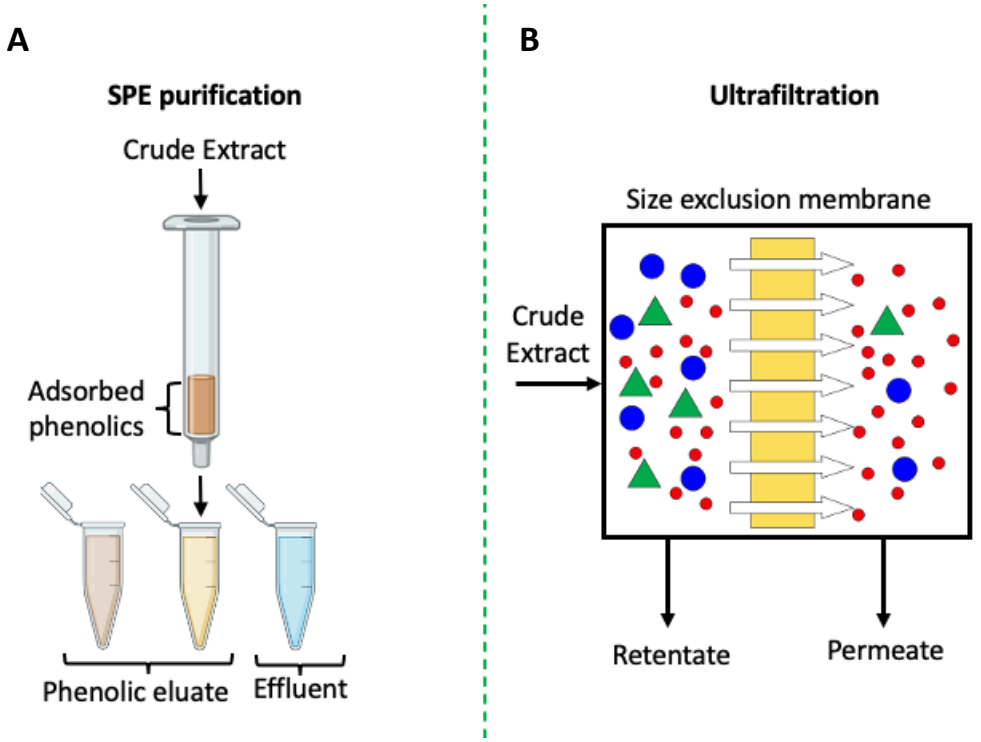


Figure 5.1: Outline of the steps for purification of phenolic extracts via adsorption (A) and ultrafiltration (B).

SPE-Solid phase extraction.

Adsorption purification involves the removal of phenolic compounds from crude extracts onto the surface of an adsorbent material, followed by their elution. Studies focused on phenolic purification from agri-food residues have increasingly adopted non-toxic elution

solvents and assessed the regenerability of both adsorbents and solvents which is critical in determining the efficiency and feasibility for the development of commercial extracts (Campone et al., 2020). To produce high-purity phenolic extracts, adsorbents must be evaluated for maximum adsorption capacity as well as their affinity and selectivity towards the phenolics in the crude extract (Yangui et al., 2017). Further optimisation is typically conducted by investigating the impacts of pH and of different elution solvents to maximise phenolic purity and process efficiency (Ge et al., 2020).

Pressure-driven membrane filtration methods, including microfiltration, ultrafiltration and nanofiltration, are well established technologies in the food and beverage industries for separating compounds predominantly on their molecular weight. These techniques differ by pore size with nanofiltration membranes retaining molecules with molecular weights between 200-1000 Da, ultrafiltration between 1000-100,000 Da whilst microfiltration typically separates between 0.1-10 µm (Conidi et al., 2018). They have also been evaluated for the sequential purification of phenolics from crude extracts, for example to extract proanthocyanidins from winery by-products (Santamaría et al., 2002). Researchers have demonstrated high antioxidant activity of the separated retentate fraction such as those from pomegranate juice separated via ultrafiltration (Conidi et al., 2017).

Chapter 4 reported the characterisation of various hop co-products based on their phenolic contents and antioxidant activities. Among these, CO_2R-HERK was identified as the most promising candidate for purification trials due to its wide availability and high concentrations of prenylflavonols. In this study the objective was to evaluate adsorption and ultrafiltration purification methods for CO_2R-HERK phenolics using 'green' solvents and techniques. The overall aim was to compare phenolic purity and antioxidant activity of the resulting hop fractions to commercial extracts from different plant sources. PAD950 and PVPP were chosen

as adsorbents whilst three ultrafiltration membranes with different molecular weight cut-offs were evaluated.

5.2-Materials

5.2.1-Chemicals

PVPP (~110 µm particle size) was obtained from Sigma Aldrich. Waters solid phase extraction (SPE) 3 ml cartridges and frits were obtained from Agilent (Santa Clara, USA). All other chemicals and reference standards used in this study were sourced according to 2.1.1.

5.2.2-Materials

CO_2R-HERK was sourced according to 2.1.2. Hop purified phenolic fractions from CO_2R-HERK were obtained from Extractis (Dury, France). An outline of the processes by which these fractions were generated is provided in section 5.3.3. For benchmarking the phenolic content of hop fractions, comparison extracts were sourced as detailed in Table 5.1.

Table 5.1: Commercial plant phenolic extracts sourced for analysis in this study.

Producer	Commercial Preparation	Plant source and composition
Givaudan-Naturex Brand (Vernier, Switzerland).	Onyxen A34	*M. glabra L.* (acerola cherry) juice powder produced by spray drying. 34% ascorbic acid.
	Green tea extract 38% polyphenols WS	Extract obtained from *C. sinensis L. Kuntze* (Green tea leaves) (38% polyphenol content).
	Overseal Carantho_OF1021	*D. carota L.* (wild carrot) anthocyanin (2.8 %) powder with maltodextrin carrier.
	Elderberry fruit powder	*S. nigra L.* (elderberry) juice powder. 4 % anthocyanidins.
	Hibiscus flowers LE WS	Liquid extract obtained from *H. sabdariffa L.* (hibiscus) flowers. 20 % total acids.
	Vegebrite Black Carrot LWS	*D. carota L.* juice concentrate.
	StabilEnhance ESR D 4	*R. officinalis L.* (rosemary) leaf extract. 4-4.5% rosmarinic acid.
Berkem (Blanquefort, France).	Vitisol	*V. vinifera L.* (grape) seed extract. (≥99% oligomeric proanthocyanidin content).
	Pineol	*P. Pinaster L.* (pine) bark extract.
	Grape OPC	*V. vinifera L.* (grape) seed extract. (≥80% oligomeric proanthocyanidin content).
Puredia (Irvine, California).	CyanthOx 50	Seabuckthorn extract (≥50% proanthocyanidin content).
	Omegia powder	Seabuckthorn extract (vitamins and omega-3-6-7-9).
Kalsec (Kalamazoo, USA).	Herbalox	Rosemary extract.
The Malt Miller (Swindon, UK).	Brewtan B	Gallotannin extract.
Labor Veritas (Zürich, Switzerland).	ICE-3	Hop bitter resin extract.

5.3-Methods

5.3.1-Preperation of non-purified hop phenolic extract

A non-purified CO_2R-HERK hop phenolic extract was prepared to evaluate degree of purification of purified fractions. In brief, 100 g milled CO_2R-HERK was extracted with 1 L 50% aqueous ethanol (v/v) (E50) using a Stuart Roller Shaker at 60 rpm for 15 min. The extract was then filtered through Whatman grade 1 filter paper using vacuum filtration, chilled overnight at 4 °C and centrifuged for 10 min at 4000 rpm after which the non-dissolvable material was discarded. Ethanol was removed using a Heidolph HeiVap rotary evaporator (Schwabach, Germany) at 25 °C for 1 h, and the resulting aqueous solution was freeze-dried for 72 h.

5.3.2-PVPP SPE Purification

5.3.2.1-Preperation of PVPP SPE columns

PVPP SPE columns were prepared using a method modified from Díaz et al. (2022). 200 mg PVPP was packed into 3 ml cartridges and contained within frits for a bed height of 8 mm.

5.3.2.2-Adsorption of hop phenolics onto PVPP

PVPP adsorption trials were carried out with CO_2R-HERK extract before and after ethanol removal. 100 g milled CO_2R-HERK was extracted with 1 L E50 and the filtered solution was chilled overnight at 4 °C and centrifuged for 10 min at 4000 rpm. Half of the recovered volume underwent ethanol removal and was centrifuged for 10 min to produce E0 and E50 extracts which were then acidified to pH 4 with 1 M HCl as performed by Magalhães et al. (2010). PVPP cartridges were loaded onto a Waters 20 position cartridge manifold, equilibrated with ethanol (5 ml) and conditioned with deionised water (5 ml). Successive cycles of hop extract were then loaded and passed through a PVPP column at 20°C at a flow rate of 1 drop/s in increments of 2.5 ml for a total of 12.5 ml/200 mg PVPP syringe (5 cycles). Syringes were treated and evaluated in triplicate for each solution.

E0 and E50 starting solution and post adsorption solutions were diluted 1 in 2 and 1 in 3 with water and E50 respectively, and analysed for total phenol content (TPC), proanthocyanidin content (PAC) and xanthohumol using methodology outlined in 2.2.4 and 2.2.6.1. Results from the TPC and PAC assays were expressed as caffeic acid equivalents (CAE) and procyanidin B3 equivalents (PB3E) respectively. Theoretical adsorption was calculated using the formula:

100 – ((mg in post adsorption solution / mg in starting solution) * 100)

An overview of the processing steps for PVPP adsorption trials is presented in Figure 5.2.

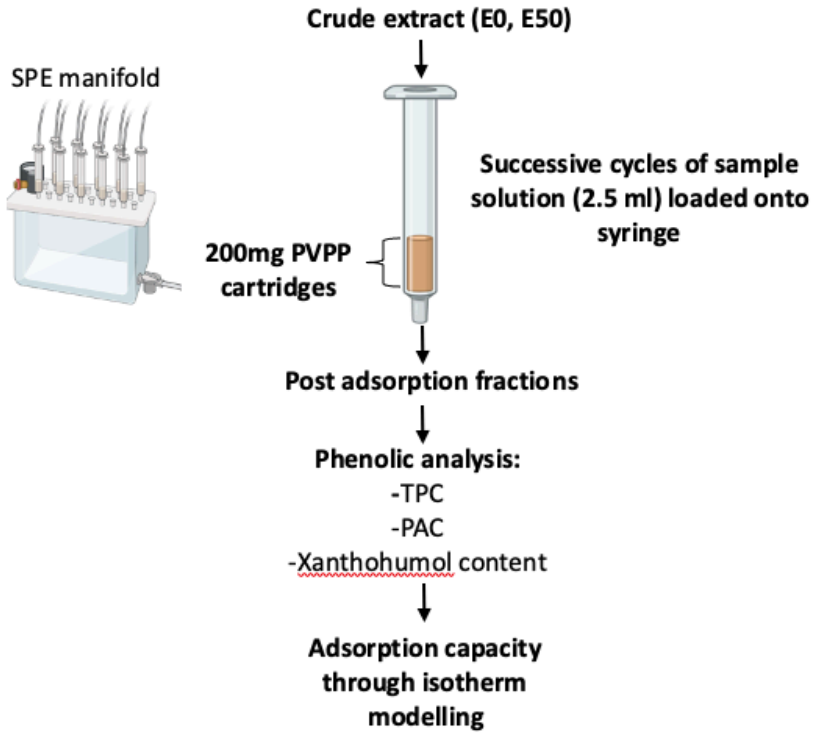


Figure 5.2: Schematic representation of the processing steps for PVPP adsorption of phenolics from CO_2R-HERK crude extracts.

TPC-Total phenol content. **PAC**-Proanthocyanidin content. **E50**-50% aqueous ethanol (v/v) extract. **E0**-Extract after ethanol removal.

5.3.2.3-Desorption of hop phenolics from PVPP

For desorption trials 100 g CO_2R-HERK was extracted with 1 L E50 and acidified using HCl to pH 4 as outlined in section 5.3.2.2. Hop extract (12.5 ml) was loaded and passed through PVPP columns at 20°C at a flow rate of 1 drop/s. The column was then washed with deionised water (5 ml) and purged with air using a vacuum pump until dry. For desorption, five solutions

were trialled: ethyl acetate, ethanol, 0.1 M ammoniacal ethanol and a stepwise desorption with increasing concentrations of ammonia and ethanol which is detailed in Table 5.2.

Table 5.2: Solutions used for stepwise desorption.

	ml	Ethanol (%)	Water (%)	Ammonia (M)
1	2.5	50	50	0
2	2.5	100	0	0
3	2.5	100	0	0.1
4	2.5	100	0	1
5	2.5	100	0	2

Desorption solution was loaded and passed through PVPP columns at 20°C at a flow rate of 1 drop/s in increments of 2.5 ml for a total of 7.5 ml/200 mg PVPP syringe for all solutions other than stepwise desorption (12.5 ml/200 mg PVPP syringe). Acetic acid was used to neutralise ammoniacal ethanolic solutions. 200 µl of eluted solution was dried down using a centrifugal evaporator at 45 °C for 2 h (preset: 3). Dry extracts were then re-solubilised with E50 and analysed for xanthohumol content and TPC expressed as CAE using methodology outlined in 2.2.4 and 2.2.6.1 respectively. An overview of the processing steps for PVPP desorption trials is presented in Figure 5.3.

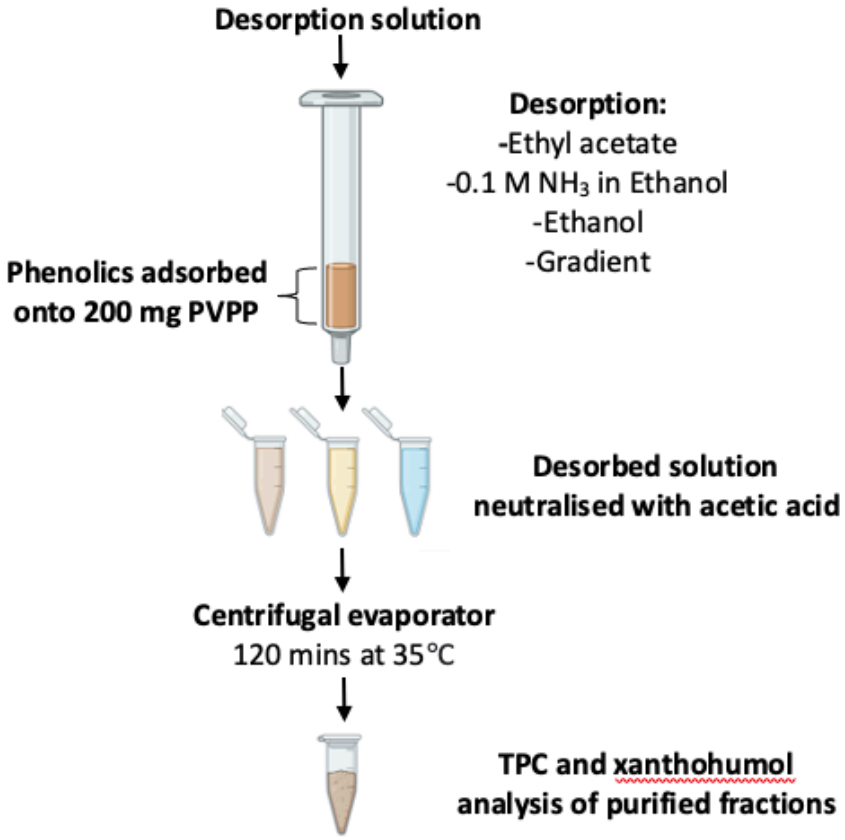


Figure 5.3: Schematic representation of the processing steps for PVPP desorption of phenolics from CO_2R-HERK crude extracts.

TPC-Total phenol content. PAC-Proanthocyanidin content.

5.3.3-Extractis trials

Further purification trials of phenolics from CO_2R-HERK were conducted by Extractis and the resulting fractions were analysed at the University of Nottingham.

5.3.3.1-Extractis-SPE Purification

For extraction, 4 kg CO_2R-HERK was mixed with 40 L E50 for 1 h at 20°C and then filtered using felt with a porosity of approximately 25 µm. Ethanol was removed using a Rotavapor R220 Büchi rotary evaporator (Schwabach, Germany) between 30-40 °C for approximately 36 h. The solution was then stored at 4 °C overnight, producing a sedimented precipitate. This precipitate was isolated via centrifugation and washed by redispersing in RO water and centrifuging twice. This fraction was then freeze-dried and stored for analysis at 4 °C.

The aqueous solution was purified using 300 g Purolite PAD950 resin in a column with a 300 ml bed volume (BV). A 3 L solution (10 BV) was loaded onto the column at 4BV/h, which was then washed with 900 ml RO water (3 BV) at 4 BV/h. Desorption was then performed with 1.5 L E50 at 4 BV/h. Ethanol was then removed from the eluates using rotary evaporation before being freeze-dried and stored for analysis at 4 °C.

5.3.3.2-Extractis-Ultrafiltration

Ultrafiltration trials were conducted on CO_2R-HERK aqueous extract post ethanol removal, the production of which is outlined in the previous section. Three membranes were evaluated: FS40, GR51 and UFX10, sourced from Alfa Laval (Lund, Switzerland). Each membrane underwent the same processing conditions generating a permeate and retentate for each membrane. Ultrafiltration was performed using a Labcell (Farnborough, UK) filtration unit with a membrane surface area of 28 cm^2 at 7 bar. An initial mass of 500 g was loaded onto the unit and the filtration process was carried out for 6 h under constant conditions with an average flow rate of 24 $L/h/m^2$. The resulting solutions were then freeze-dried and stored at 4 °C for analysis.

Figure 5.4 presents an overview of the processing steps performed by Extractis, as well as the phenolic fractions generated from the ultrafiltration and SPE purification of CO_2R-HERK phenolics.

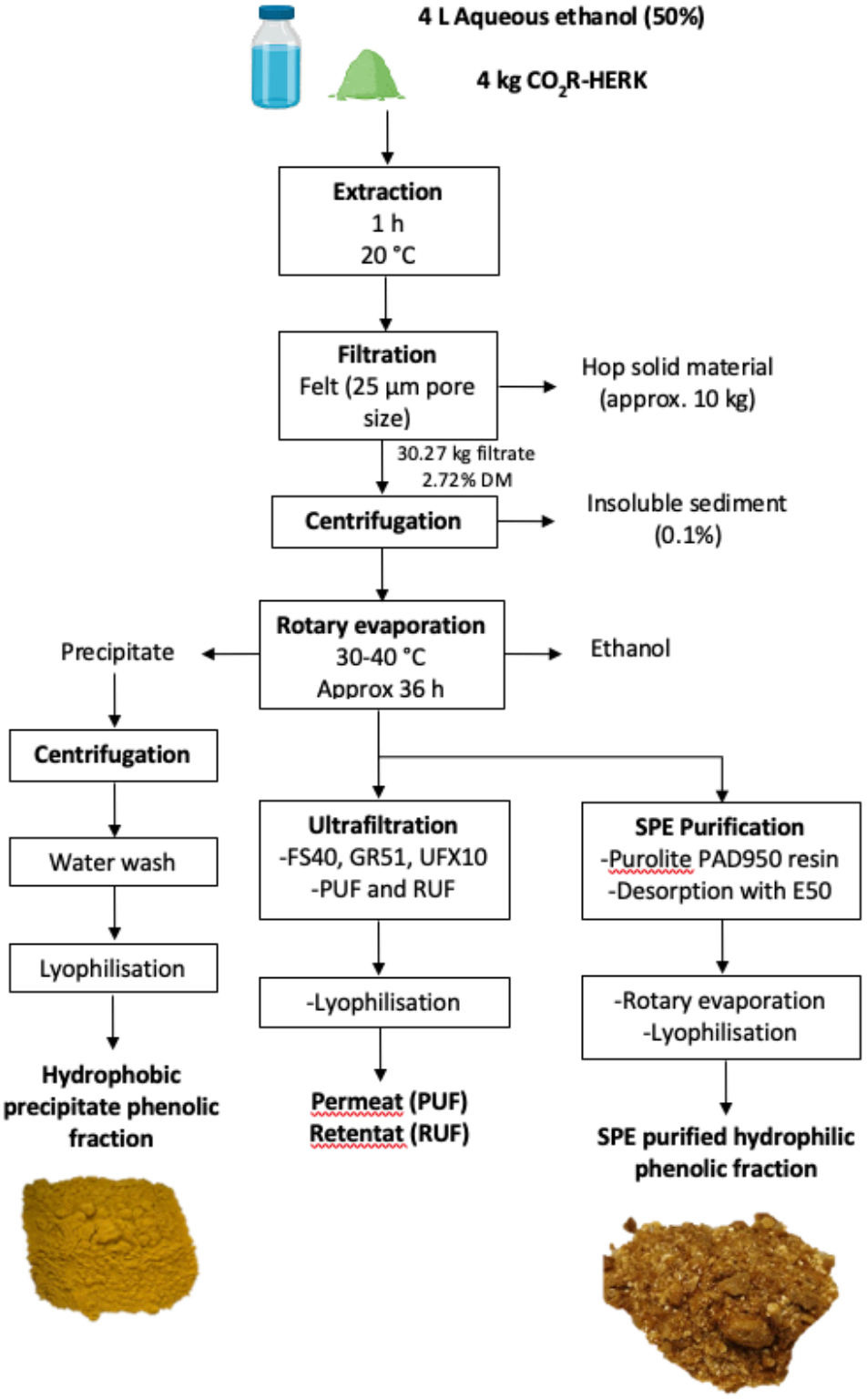


Figure 5.4: Schematic representation of the processing steps performed by Extractis, as well as the phenolic fractions generated from the ultrafiltration and SPE purification of CO_2R-HERK extract.

FS40-Polypropylene fluoropolymer membrane with a molecular weight cut-off (MWCO) of 100 KDa. **GR51**-Polypropylene fluoropolymer membrane with a MWCO of 50 KDa. **UFX**-Polypropylene fluoropolymer membrane with a MWCO of 10 KDa.

5.3.4-Spectrophotometric determination of phenolic content

The phenolic content of extracts was measured using the TPC and PAC assays with results expressed as caffeic acid equivalents (CAE) and procyanidin B3 equivalents (PB3E) respectively, using methodology outlined in 2.2.4. Extracts were analysed in triplicate at 0.5 mg/ml and 5 mg/ml respectively for TPC and PAC assays.

5.3.5-Antioxidant activity of extracts

The antioxidant activity of extracts was analysed using the DPPH, FRAP and ORAC assays and resulting data was expressed as Trolox equivalents as detailed in 2.2.5. For DPPH radical scavenging activity % (RSA) extracts were solubilised in E50 and analysed at multiple concentrations in triplicate between 1-120 µg/ml to calculate IC50 values. The DPPH RSA of each extract analysed at different concentrations is provided in Appendix 5, Figure 2. For FRAP analysis, extracts were solubilised in E50 and analysed at four concentrations in triplicate to determine the working linear range (see Appendix 5, Figure 3). Trolox equivalents were calculated based on analysis at 0.1mg/ml E50 for all materials except for xanthohumol and ProXantho which were calculated at 0.2 mg/ml E50 due to lower activity. For ORAC analysis, extracts were solubilised in PBS (pH 7.4) or aqueous acetone 50% (v/v) depending on the hydrophilicity of the extract and analysed at multiple concentrations in triplicate to determine the working linear range (see Appendix 5, Figure 4). ORAC Trolox equivalents were calculated based on analysis at 0.0025 mg/ml for all materials.

5.3.6-Chromatographic analysis of extracts

For the quantitation of xanthohumol, α and β-acids in hop fractions, HPLC-DAD was used following methodology outlined in 2.2.6.1. Extracts were solubilised in E50 and analysed in triplicate at 0.5 mg/ml.

For phenolic screening and quantitation, LC-ESI-qTOF-MS/MS was used following the methodology outlined in 2.2.6.2. Extracts were solubilised in E50, syringe filtered at 0.22 µm

and analysed at 0.5 mg/ml in triplicate. 4-methyl catechol was used as the internal standard (IS) at a final concentration of 2.5 µg/ml. For quantitation, standards were solubilised in E50, diluted across 4 orders of magnitude (see Appendix 4, Table 1) and analysed in triplicate at the beginning, middle and end of the run. Quantitative analysis was performed as outlined in 4.3.2 using standard curves detailed in Appendix 4, Table 1. Semi-quantitation was performed using standard curves from structurally similar standards where available. For phenolic screening, peak areas were normalised to IS.

5.4-Results and Discussion

5.4.1-Hop phenolic purification

Research reported in this chapter aimed to evaluate purification techniques for the isolation of these compounds to produce an extract with maximal antioxidant activity. As such three phenolic purification techniques were evaluated: PVPP SPE, Purolite PAD950 SPE and ultrafiltration. For comparison, a non-purified freeze-dried extract was generated to evaluate degree of purification achieved by each method.

5.4.2-PVPP SPE Phenolic Purification

Polyninylpolypyrrolidone (PVPP) is an inert and insoluble cross-linked polymer commonly used in the beverage industry to clarify and stabilise beer and wine by removing polyphenols (Laborde et al., 2006). Recently there has been growing interest in its application for purifying phenolics from crude extracts due to its high phenolic specificity and good regenerability (Díaz et al., 2022). As such, phenolic fractions generated using PVPP from various plant sources have been evaluated for a range of bioactive properties, including chemopreventive (Gerhäuser et al., 2002), neuroprotective (Rocha et al., 2020) and antioxidant activity (Ferreira et al., 2018). As such, a recent review on recycling phenolics from brewery by-products highlights PVPP as a promising material for hop phenolic purification (Silva et al., 2023). PVPP adsorbs polyphenols through hydrogen bonding with selectivity increasing with

number of aromatic rings, hydroxylation and oligomerisation, however at higher concentration removes all polyphenols (McMurrough et al., 1995).

5.4.2.1-Adsorption

PVPP adsorption trials were carried out with CO_2R-HERK extract before and after ethanol removal (E50 and E0). Biendl (2012) highlighted that ethanolic solutions can be treated with PVPP (unlike non-polar adsorption resins) which presents the possibility of purifying hydrophobic prenylflavonoids and hydrophilic phenolics in the same step without the need for ethanol removal. However other studies have reported higher binding affinities in water compared to aqueous methanol (Loomis and Battaile, 1966, Andersen and Sowers, 1968). Therefore, in this study both E50 and E0 crude extracts were evaluated for phenolic adsorption onto PVPP. Experiments were conducted at 20 °C as this has shown to provide higher adsorption capacities for catechins onto PVPP than at higher temperatures (40, 60 °C) (Dong et al., 2011). Extracts were acidified to pH 4 with HCl as performed by Magalhães et al. (2010), which is sufficiently low enough to suppress ionization of phenolic hydroxyl groups. Crude extracts were passed through a PVPP column, and pre- and post-adsorption solutions were analysed for TPC, PAC and xanthohumol content to calculate theoretical adsorption. E0 extract was not evaluated for xanthohumol adsorption onto PVPP as it was only present at trace level in the starting solution (data not shown). Appendix 5, Figure 1 presents LC-DAD chromatograms illustrating the difference in xanthohumol content in the E50 extract before and after PVPP treatment. Figure 5.5 presents theoretical adsorption of phenolics for both extracts evaluated. Since TPC, PAC and xanthohumol contents were present at different concentrations within each solution, calculating adsorption per ml extract provides a more accurate measurement of adsorption affinity compared to calculating based on the weight of phenolic added.

Adsorption affinity was in order of xanthohumol > PAC > TPC for E50 extract, whilst TPC and PAC were very similar for E0 extract. This is in line with the results of Magalhães et al. (2010) who investigated PVPP for the isolation of hop phenolics and found the highest adsorption capacity and recovery yields for prenylflavonoids such as xanthohumol. The comparatively lower adsorption of TPC compared to xanthohumol for the E50 extract may be due to bitter resins not adsorbing onto PVPP but interacting with Folin reagent or lower affinity of glycosidically bound phenolics such as flavonol and phloroglucinol glycosides. Phloroglucinol and flavonol glycosides were identified in CO_2R-HERK in Chapter 4 and have shown to have lower affinity to PVPP than their respective aglycones (Magalhães et al., 2010). Similarly, Laborde et al. (2006) highlighted that adsorption affinity between PVPP and aglycones in wine was 4-5 times higher than for the respective 3-O-glucosides, which is likely explained by the sugar moiety reducing the interaction forces.

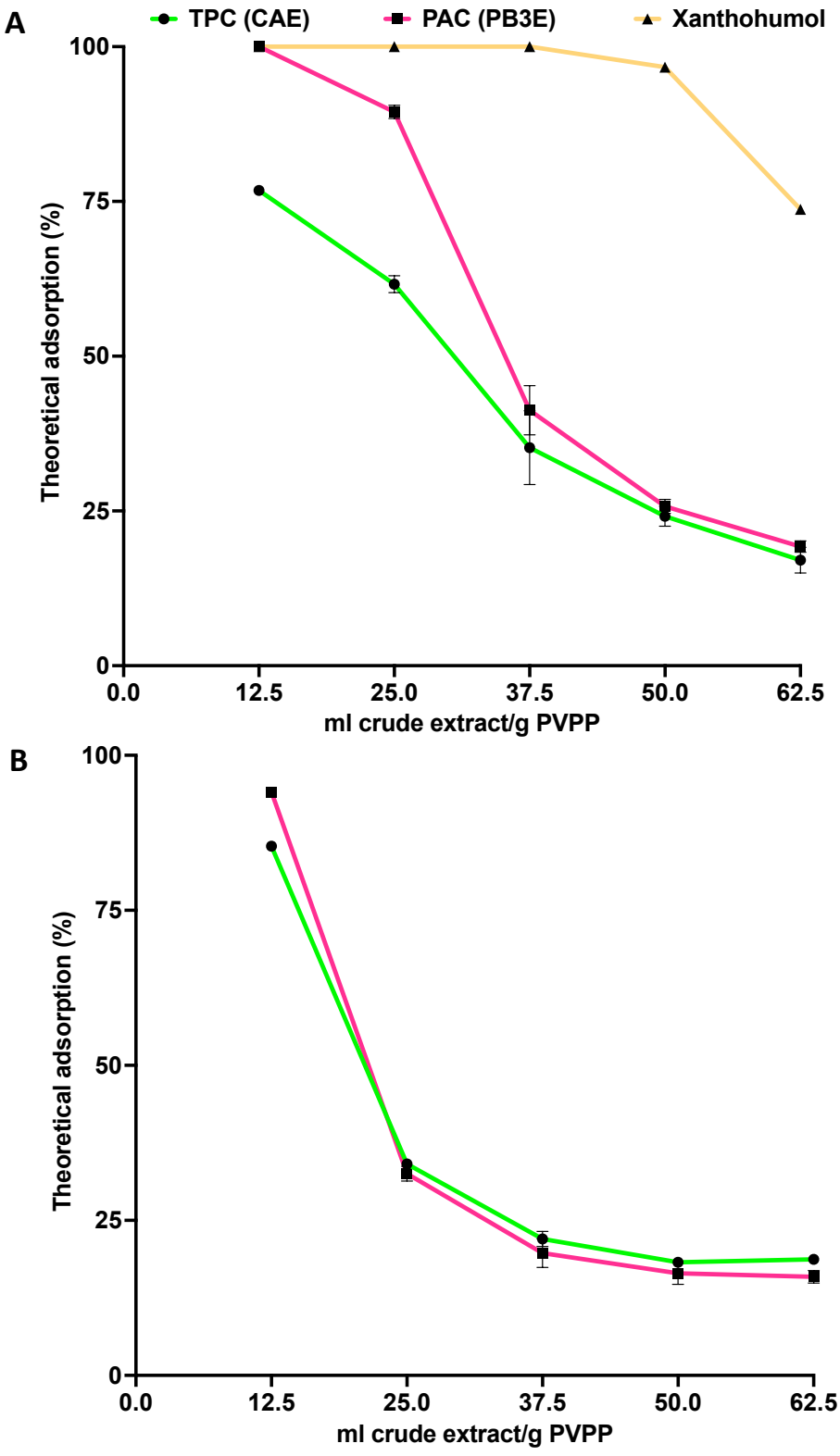


Figure 5.5: Theoretical adsorption of total phenol content, proanthocyanidin content and xanthohumol onto PVPP calculated for E50 (A) and E0 (B) crude extracts of CO_2R-HERK.

CAE-Caffeic acid equivalents. **PB3E**-Procyanidin B3 equivalents. **E50**-50% aqueous ethanol (v/v) extract. **E0**-Extract after ethanol removal.

5.4.2.2-PVPP phenolic adsorption Isotherms

For adsorption, components of the crude extract are adsorbed onto PVPP until dynamic equilibrium is reached. Adsorption isotherms are used to characterise the equilibrium, with the Langmuir and Freundlich adsorption the most commonly adopted models for phenolic adsorption (Kalam et al., 2021). The Langmuir isotherm assume a homogenous surface with a finite number of sites whilst the Freundlich isotherm assumes a heterogenous surface and allows for multi-layer adsorption. In this study, the Langmuir isotherm was applied as it has shown by Qian et al. (2023) and Folch-Cano et al. (2013) to have a better fit for adsorption of phenolics from crude extracts onto PVPP. For this, mg added/g PVPP was plotted against mg adsorbed/g PVPP for TPC, PAC and xanthohumol, and the Langmuir model was applied (Figure 5.6) using the equation:

$$\frac{q_e}{q_m} = \frac{K_L C_e}{1 + K_L C_e}$$

q_e: amount of adsorbate adsorbed per unit mass of adsorbent

q_m: maximum adsorption capacity

K_L: langmuir constant

C_e: equilibrium concentration of the adsorbate

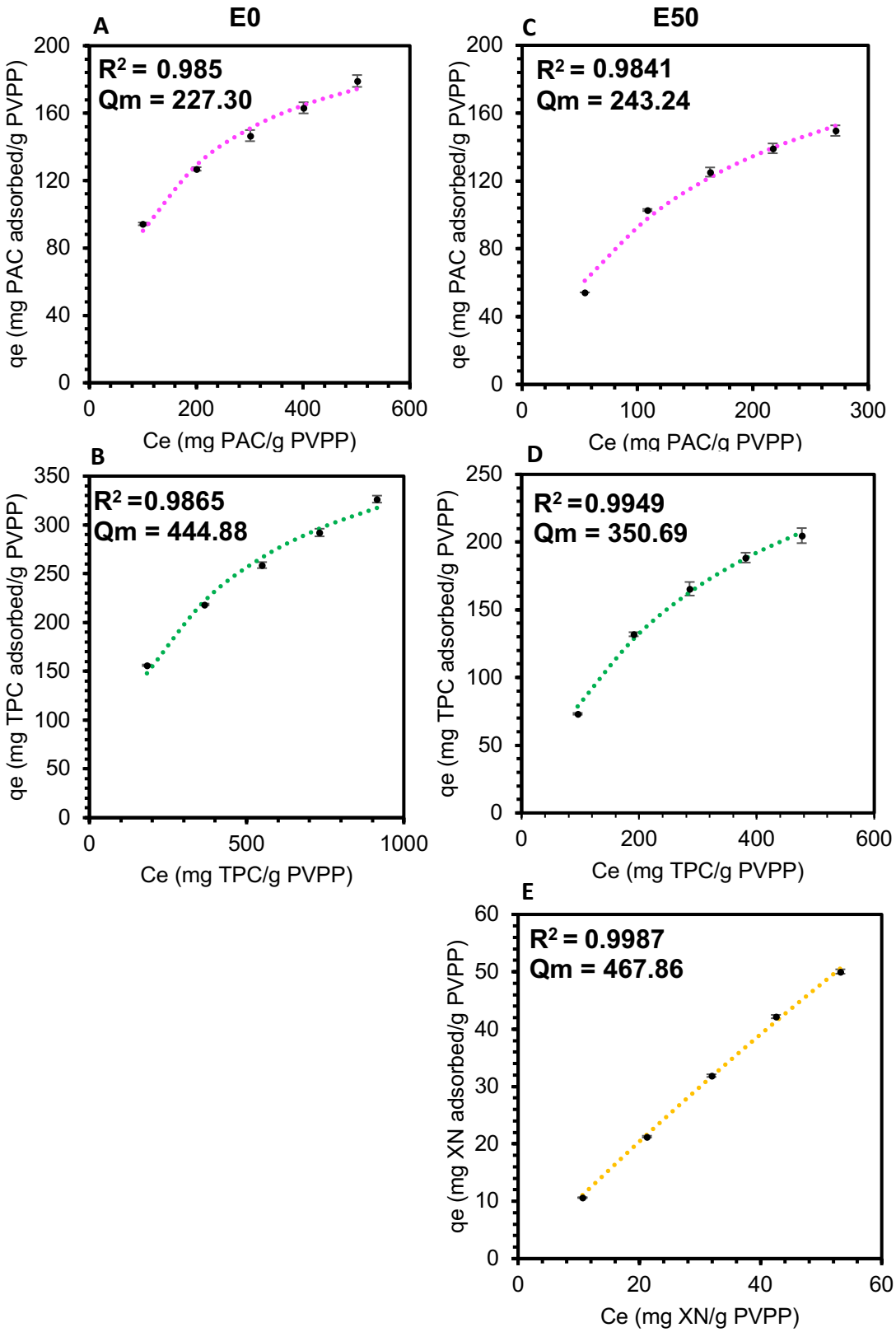


Figure 5.6: Langmuir adsorption isotherms of proanthocyanidin content (PAC), total phenol content (TPC) and xanthohumol onto PVPP for E0 (A-B) and E50 (C-E) CO_2R-HERK crude extract.

Qm: maximum adsorption capacity (mg/g PVPP). **E50**-50% aqueous ethanol (v/v) extract. **E0**-Extract after ethanol removal.

The R^2 values generated from Langmuir isotherm models were high (>0.98) for all assays for both solutions indicating that adsorption behaviour was consistent with monolayer adsorption. Maximum adsorption capacity (Qm) is characterised by a saturation point (plateau) where no further adsorption can take place as all sites are occupied. Qm values were in the order of xanthohumol > TPC > PAC for E50 solution, and TPC > PAC for E0 solution. PAC Qm was slightly higher for E50 solution whereas TPC was higher for E0 solution. As mentioned in 5.4.2.1, the lower TPC Qm for E50 compared to E0 may be due to the impact of bitter resins which do not adsorb onto PVPP but do interact with Folin reagent (Dadic and Lavallee, 1983). TPC Qm was lower than reported by Dong et al. (2011) who reported a total catechins Qm of 671.77 mg/g PVPP. However, the Qm's reported in this study for phenolic adsorption onto PVPP are generally higher than those reported for other adsorption resins.

The use of crude extracts in adsorption analysis represents both an advantage and a limitation of this study. Adsorbent performance observed with single-compound model solutions has shown to not accurately represent that of crude extracts due to interactions between phenolics during adsorption in multi-compound mixtures (Kammerer et al., 2010). Therefore, in this study crude extracts were evaluated instead of model solutions to better reflect conditions relevant for industrial applications. However, the extracts evaluated (E50, E0) differed in ethanol content as well as phenolic content which makes it challenging to isolate the effect of ethanol % on phenolic adsorption and to compare the PVPP affinities of different phenolics. When comparing PVPP adsorption of phenolics between both extracts (E0, E50), E50 was more promising due to the high Qm of xanthohumol, a major phenolic compound of CO_2R-HERK. Therefore, E50 extract was chosen for subsequent desorption trials.

5.4.2.3-Desorption

A potential challenge of using PVPP for polyphenol purification is the need for efficient desorption of phenolics with a high recovery rate using 'green' solvents. Most trials on

desorption from PVPP have adopted toxic solvents such as 70% aqueous acetone (v/v) (Magalhães et al., 2010), or methanol (Lindemann et al., 2020), which are effective for hop phenolics but unsuitable for developing food-grade extracts. Gerhäuser et al. (2002) used sodium hydroxide (pH 13.5) for desorption of phenolics from PVPP used to treat beer, with the solution subsequently neutralised to pH 3.5 with HCl. According to a Heineken patent on regenerating PVPP after beverage stabilisation (Noordman et al., 2016), an aqueous regeneration liquid with a pH of at least 10.0 (using sodium hydroxide) at temperatures between 40-80 °C can regenerate PVPP over 20 times. However environmental impact and degree of phenolic oxidation using this method isn't known, as recovery of phenolics was not the aim of the patent.

Ferreira et al. (2018) investigated recovery of phenolics from PVPP from wine fining using 'green' solvents. Desorption used an ammoniacal solution of ethanol (0.1 M) subsequently neutralised with acetic acid, which achieved recovery rates of 80-90%. Ammonia is suited for recovery due to high alkalinity and volatility, conditions which are necessary for phenolic desorption and drying down extracts respectively. Similarly, Biendl (2012) investigated phenolic desorption from PVPP and found that ethyl acetate was effective for selective desorption of xanthohumol, which is the principle phenolic compound for CO_2R-HERK as highlighted in Chapter 4. Considering these findings, this study aimed to evaluate ethyl acetate, ethanol and ammoniacal ethanol (0.1 M-2 M) for the desorption of phenolics adsorbed onto PVPP. CO_2R-HERK E50 extract was loaded onto syringes containing PVPP, and theoretical adsorption/g PVPP of TPC and xanthohumol was calculated for each syringe as outlined in 5.3.2.2. No xanthohumol was detected in any post-adsorption solutions as shown in chromatograms presented in Appendix 5, Figure 1. PVPP cartridges were washed with UP water, and desorbing solution was passed through the PVPP cartridges in successive cycles of 2.5 ml. Resulting fractions were dried using a centrifugal evaporator, resolubilised in E50 and analysed for TPC and xanthohumol. The amounts of TPC and xanthohumol desorbed from

200 mg PVPP for the different treatments is presented in Figure 5.7, and the recovery yields are presented in Table 5.3.

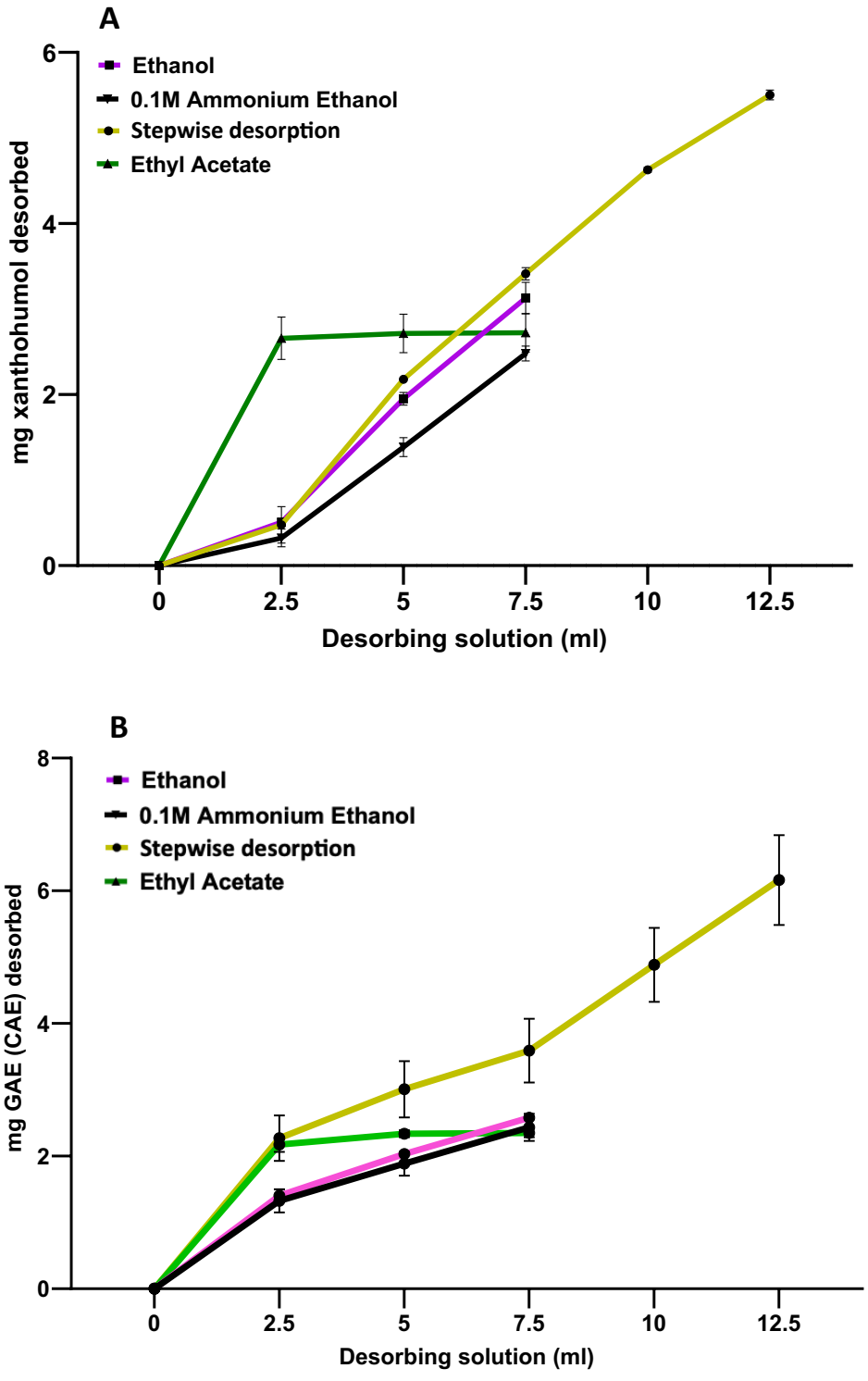


Figure 5.7: Amount of xanthohumol (A) and total phenol content (B) desorbed from 200 mg treated PVPP from the different treatments.

CAE-Caffeic acid equivalents. **Stepwise desorption**-Increasing proportion of ethanol and/or ammonia.

Table 5.3: Recovery yields (%) of xanthohumol and TPC desorbed by the different treatments.

	Desorbing volume (ml)	Xanthohumol	TPC (CAE)
Ethyl acetate	**2.5**	34.03 ± 2.74	6.27 ± 0.23
	5	34.76 ± 2.49	6.73 ± 0.11
	7.5	34.85 ± 2.50	6.77 ± 0.13
Ethanol	**2.5**	6.51 ± 0.49	4.05 ± 0.19
	5	24.97 ± 0.82	5.86 ± 0.12
	7.5	40.06 ± 2.04	7.43 ± 0.05
Ammonia ethanol (0.1 M)	**2.5**	4.14 ± 1.61	3.82 ± 0.36
	5	17.74 ± 1.73	5.43 ± 0.37
	7.5	31.75 ± 1.36	7.01 ± 0.42
Stepwise desorption	**2.5**	6.10 ± 2.36	6.54 ± 0.70
	5	27.88 ± 0.18	8.67 ± 0.86
	7.5	43.69 ± 0.78	10.34 ± 0.98
	10	59.25 ± 0.26	14.07 ± 1.14
	12.5	70.47 ± 0.61	17.76 ± 1.83

CAE-Caffeic acid equivalents. **Stepwise desorption**-Increasing proportion of ethanol and ammonia.

Recovery rates for xanthohumol were higher than TPC which was particularly low (<20%) for all treatments. Ethyl acetate was most effective for xanthohumol desorption for the first 2.5 ml however the second and third cycles recovered very little. This may indicate a channelling effect in part due to its low viscosity which may limit PVPP contact, even with a controlled flow rate of 1 drop/s. TPC recovery was greater for higher ammonia solutions (1, 2 M), however there was no difference between ethanol and 0.1 M ammonia ethanol. Recovery rates of TPC were much lower than those reported by Ferreira et al. (2018), Mendes et al. (2018) and Rocha et al. (2020) who achieved recovery rates of 80-90% for wine phenolics using 0.1 M ammonia ethanol. They were also lower than those reported by Jankowiak et al. (2015) and Xu et al. (2005) for isoflavones using only ethanol for desorption. This seems unlikely to be related to differences in affinity to PVPP between wine and hop phenolics, as in each case catechins and B-type procyanidins make up a large portion of the phenolic fraction of both materials (Garrido and Borges, 2013).

A key difference between this study and others that report high phenolic recovery yields using PVPP is the method. Whilst this study used fixed-bed PVPP SPE columns, other studies used

'free' PVPP agitated in solution. PVPP columns provide several advantages, such as a higher concentration gradient, reduced saturation effects, and improved scalability. However, the fixed column setup reduces contact time between PVPP and desorbing solution which may limit phenolic desorption, depending on phenolic affinity to PVPP. For example, Ferreira et al. (2018) and Magalhães et al. (2010) achieved high phenolic recovery by agitating 'free' PVPP for 15 min during desorption, with Magalhães et al. (2010) incorporating sonication as well. Scoma et al. (2012) evaluated Amberlite XAD16 as a solid phase and found that only 58% phenolic recovery was achieved in 15 min, with 90 min necessary for complete desorption. This suggests that hop phenolics, especially those which interact strongly with Folin reagent, may require longer contact times for optimal phenolic recovery. Future research should explore longer contact times using 'free' PVPP or a recirculating fixed-bed column setup to improve phenolic recovery during desorption which has been shown to be effective by Frascari et al. (2016) for olive mill phenolic purification.

5.4.3-Extractis-Ultrafiltration and SPE phenolic purification

To evaluate SPE and ultrafiltration as hop phenolic purification techniques, trials were conducted at Extractis generating freeze-dried fractions which were analysed at the University of Nottingham. For SPE trials, 2 fractions were generated, a hydrophobic precipitate isolated via centrifugation after ethanol removal, and a hydrophilic phenolic fraction generated by desorption of loaded Purolite PAD950 resin with E50 (Figure 5.4). Chapter 4 highlighted the high concentrations of hydrophobic prenylflavonoids and bitter resins in CO_2R-HERK and therefore this precipitate fraction was of interest and evaluated. Purolite PAD950 is a polymethacrylic commercial adsorbent resin that is used for phenolic purification due to its high adsorption capacity, desorption efficiency, low cost and suitability for large scale production (Yang et al., 2016, Flórez-Fernández et al., 2019). PAD950 has been shown to be effective for the recovery of phenolics from a range of materials such as olive tree leaves (Mir-Cerdà et al., 2024) and lemon peel (Lorca et al., 2023). E50 was chosen for

desorption as it has been shown to have the highest desorption efficiency for PAD950 for the recovery of phenolics in olive oil wastewater (Cifuentes-Cabezas et al., 2022).

For ultrafiltration 3 polypropylene fluoropolymer membranes were evaluated: FS40, GR51 and UFX. The membranes evaluated had different molecular weight cut-offs (MWCO) of 100 KDa, 50 KDa and 10 KDa for FS-40, GR-51 and UFX respectively. These membranes have primarily been evaluated for their efficacy in removing impurities from juices such as suspended solids and HMW tannins from pomegranate (Hidalgo et al., 2024) and sugarcane (Saha et al., 2006) juice. In this study each membrane generated a permeate (PUF) which passed through the membrane, and a retentate (RUF) which did not. Freeze dried samples were analysed for TPC and PAC and compared to respective non-purified freeze-dried extract to assess degree of purification. The TPC and PAC of the resulting fractions is presented in Figure 5.8.

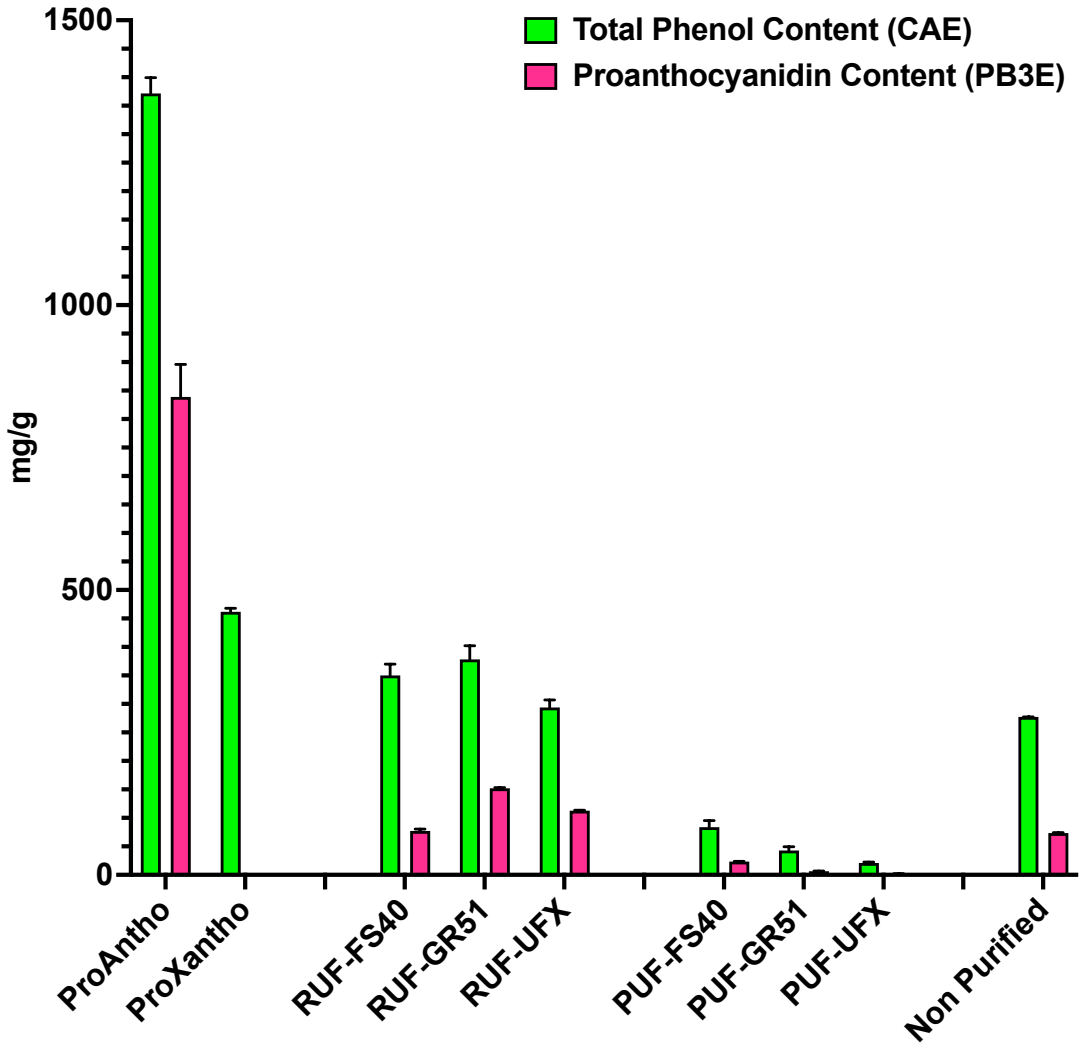


Figure 5.8: Total phenol and proanthocyanidin contents of hop fractions produced from SPE purification, precipitation, ultrafiltration and without purification.

Data represent the mean ± standard deviation of triplicate analytical measurements (n=3).

CAE-Caffeic acid equivalents. **PB3E-**Procyanidin B3 equivalents. **ProAntho-**PAD950 purified phenolic extract from CO_2R-HERK. **ProXantho-**Precipitate fraction isolated after ethanol removal. **Non-purified-**Freeze dried extract after ethanol removal. **RUF**-Ultrafiltration retentate. **PUF**-Ultrafiltration permeate. **FS40**-Polypropylene fluoropolymer membrane with a molecular weight cut-off (MWCO) of 100 KDa. **GR51**-Polypropylene fluoropolymer membrane with a MWCO of 50 KDa. **UFX**-Polypropylene fluoropolymer membrane with a MWCO of 10 KDa.

ProAntho had the highest TPC and PAC of all fractions with degrees of purification of 4.95 and 11.40 respectively. ProXantho contained no proanthocyanidins but had a higher TPC compared to ultrafiltration and non-purified fractions. This indicates that PAD950 SPE was the most effective phenolic purification technique evaluated, especially for proanthocyanidins. The proportion of PAC to TPC for ProAntho was 61.18% which is generally in line with the PAC ratio of 77% reported by Wang et al. (2014) for SP850 purified hop cone extract. However, it

was higher than the PAC ratio of CO_2R-HERK crude extract detailed in Chapter 4, most likely due to the removal of hydrophobic phenolics during ethanol removal.

The retentate fractions produced from ultrafiltration had a higher TPC and PAC compared to respective permeate fractions. This indicates that phenolics were concentrated via the passing of non-phenolic lower molecular weight compounds through the filter into the permeate. GR51 (50 KDa) was the most effective membrane for enriching phenolics in the retentate, especially for proanthocyanidin purification. This suggests that CO_2R-HERK proanthocyanidins are predominantly in the size range 50-100 KDa. The lower PAC of RUF-FS40 and correspondingly higher PUF-FS40 suggests proanthocyanidins between 50-100 KDa are lost through passing into the PUF-FS40. For all membranes evaluated, degree of purification of the retentate was low, ranging from 1.06-1.37 for TPC and 1.05-2.06 for PAC. Zagklis and Paraskeva (2015) also found that ultrafiltration exhibited a lower efficiency in phenolic purification compared to adsorption. This was attributed to poor separation of carbohydrates which have a similar molecular weight range to that of phenolic compounds. This suggests a lower selectivity of phenolic separation based on molecular weight compared to the adsorbent resins with higher specificity for phenolics through hydrophobic interactions and hydrogen bonding (Pérez-Larrán et al., 2018). Phenolic targets for purification in CO_2R-HERK differ in molecular weight, ranging from 290.26 Da (catechin) to possibly approaching 100 kDa for polymeric proanthocyanidins such as those reported in Saaz hop cones (Gadon et al., 2019a). Similarly, crude plant extracts contain significant impurities, such as high molecular weight solutes, which may limit the selectivity of ultrafiltration for phenolics (Cassano et al., 2018). It therefore seems that PAD950 is a more effective, albeit more expensive, method for CO_2R-HERK phenolic purification compared to ultrafiltration due to its higher selectivity.

To compare hop fraction phenolic content with commercially available products, plant and phenolic extracts were sourced as detailed in Table 5.1. These extracts were analysed for TPC and PAC which is presented in Figure 5.9. Commercial extracts were categorised according to their advertised contents into proanthocyanidin extracts, phenolic extracts, and plant extracts. The purification techniques for proanthocyanidin and phenolic extracts are generally not disclosed by manufacturers, however for those that are, they typically involve solvent extraction followed by adsorption. For example CyanthOx 50 is produced using hot water extraction of sea buckthorn followed by AB-8 macro-porous adsorption, desorption with E30 and spray drying (Zhu et al., 2021).

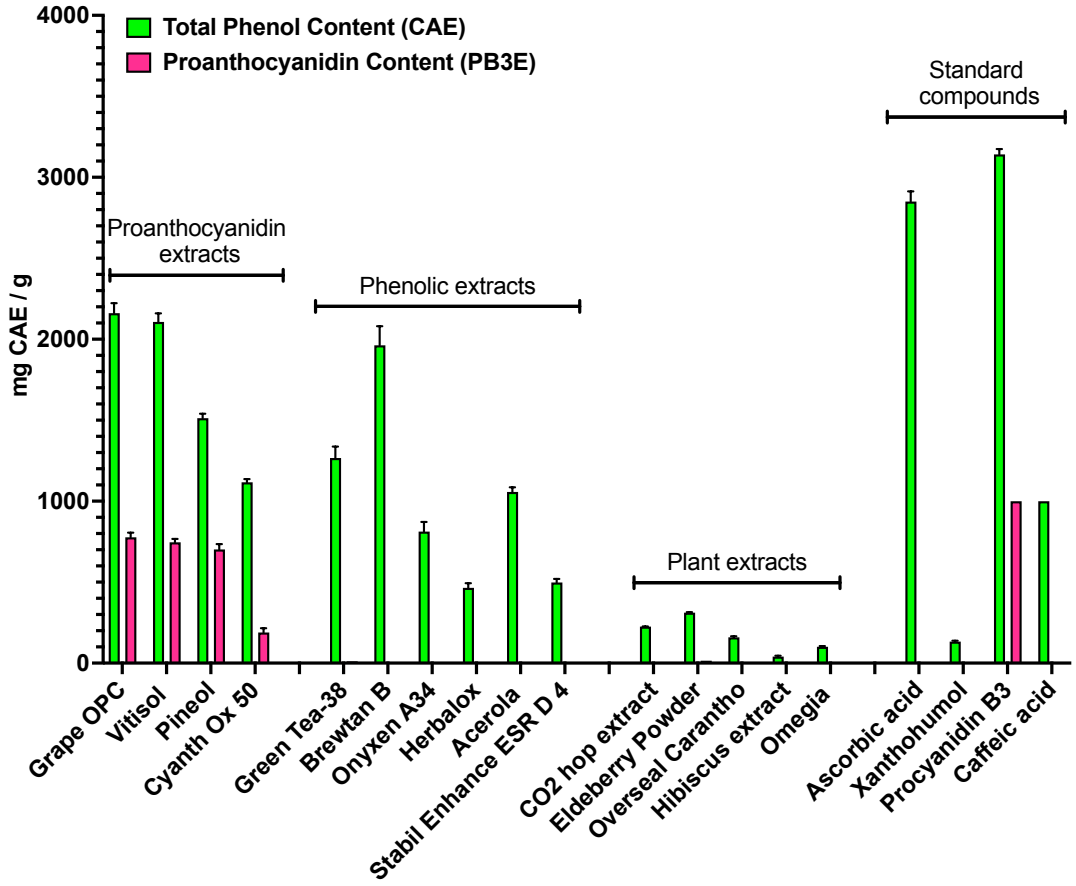


Figure 5.9: Total phenol content (TPC) and proanthocyanidin content (PAC) of commercial extracts and standard compounds. The sourcing of the commercial extracts sourcing and their advertised composition is detailed in Table 5.1.

Data represent the mean ± standard deviation of triplicate analytical measurements.

CAE-Caffeic acid equivalents. **PB3E-**Procyanidin B3 equivalents.

As expected, TPC was highest for phenolic and proanthocyanidin extracts in comparison to plant extracts. Among all the extracts analysed, Berkhem extracts (Grape OPC, Vitisol and Pineol) contained the highest PAC and TPC (along with Brewtan B). These commercial extracts are derived from bark and grape seed/skin which are a rich source of B-type oligomeric proanthocyanidins (Weber et al., 2007). Grape OPC and Vitisol have been extensively studied as natural food stabilising agents due to their high proanthocyanidin content (Ferrando et al., 2011, Rózek et al., 2010). The ProAntho fraction from CO_2R-HERK had a higher PAC (Figure 5.8) than all analysed commercial extracts (Figure 5.9), whilst its TPC was comparable to Pineol but lower than that for Grape OPC, Vitisol and Brewtan B. This indicates that ProAntho is a rich source of phenolics, and that PAC constitute a greater proportion of TPC in this sample compared to the commercial extracts. The TPC of ProXantho was higher than all plant extracts but generally lower than for phenolic and proanthocyanidin extracts. Xanthohumol was the principle hydrophobic phenolic quantified in CO_2R-HERK as outlined in Chapter 4. Xanthohumol exhibited poor interaction with the Folin-Ciocalteu reagent compared to procyanidin B3 and caffeic acid as highlighted in Figure 5.9. Therefore, chromatographic techniques are required for a better understanding of the phenolic content of ProXantho.

5.4.4-Screening and quantitative analysis of phenolics and resins

To investigate differences in phenolic and resin composition of the purified extracts, quantitation and relative quantitation analysis was performed using LC-MS and LC-DAD. ProAntho and ProXantho were chosen for further characterisation as their phenolic content was higher than ultrafiltration purified fractions (Figure 5.8). Among the commercial extracts Grape OPC, Pineol, Vitisol and CyanthOx 50 were chosen due to their higher PAC levels, making them suitable benchmarks for ProAntho. Figure 5.10 presents the relative quantitation of all identified phenolics and resins (outlined in 2.2.6.1) as a normalised heat map with samples and variables ordered according to AHC analysis. Table 5.4 presents the concentrations of phenolic and resin components quantified or semi-quantified using LC-MS

and LC-DAD. Compounds < LOQ for all materials were hesperidin, benzoic acid, trans-3-hydroxycinnamic acid, sinapic acid and procyanidin A2.

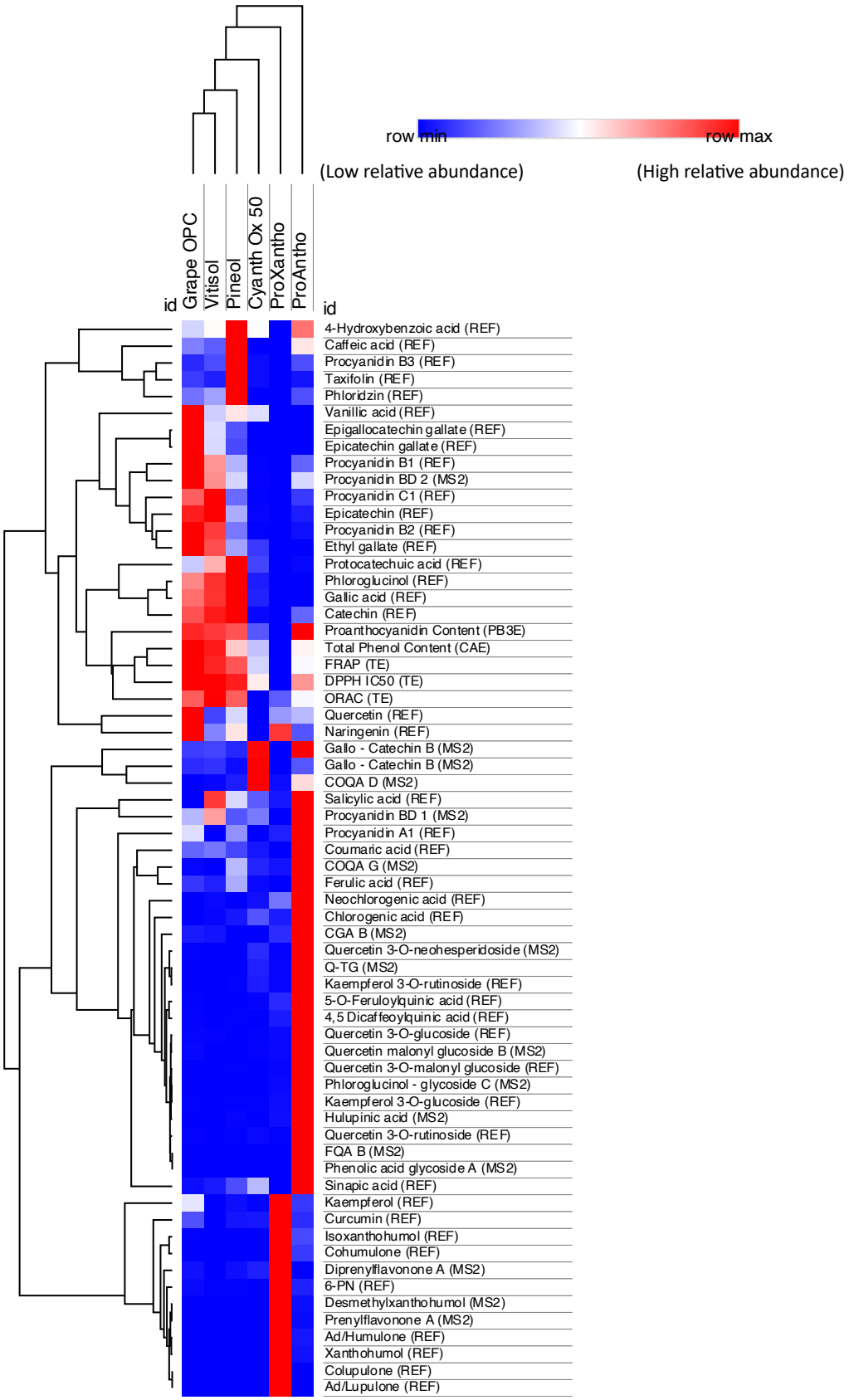


Figure 5.10: Normalised heat map of phenolic compound concentrations in hop and comparison materials with variables ordered according to AHC analysis (Euclidean distance).

ProAntho-PAD950 purified phenolic extract from CO_2R-HERK.

ProXantho-Precipitate fraction isolated after ethanol removal.

	Grape OPC	Vitisol	Pineol	CyanthOx 50	ProAntho	ProXantho
Phenolic and chlorogenic acids						
Gallic acid	83.62 ± 0.95	95.90 ± 2.10	106.35 ± 1.42	7.18 ± 0.79	< LOQ	< LOQ
Protocatechuic acid	2.38 ± 0.05	3.91 ± 0.13	5.94 ± 0.24	0.85 ± 0.04	0.15 ± 0.01	< LOQ
4-Hydroxybenzoic acid	0.54 ± 0.19	0.67 ± 0.06	1.37 ± 0.05	0.66 ± 0.07	1.04 ± 0.05	< LOQ
Caffeic acid	0.66 ± 0.14	0.40 ± 0.09	3.58 ± 0.44	< LOQ	1.83 ± 0.07	< LOQ
Vanillic acid	0.35 ± 0.12	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
Coumaric acid	1.20 ± 0.05	1.37 ± 0.04	0.89 ± 0.05	0.39 ± 0.02	5.39 ± 0.22	0.17 ± 0.04
Ferulic acid	< LOQ	< LOQ	< LOQ	< LOQ	2.96 ± 0.23	< LOQ
Chlorogenic acid	< LOQ	< LOQ	< LOQ	< LOQ	1.43 ± 0.02	< LOQ
Neochlorogenic acid (CQAE)	< LOQ	< LOQ	< LOQ	< LOQ	1.49 ± 0.05	0.15 ± 0.01
CQA B (CQAE)	< LOQ	< LOQ	< LOQ	< LOQ	1.74 ± 0.08	< LOQ
4,5-Dicaffeoylquinic acid (3,4-DCQAE)	< LOQ	< LOQ	< LOQ	< LOQ	17.46 ± 0.18	0.78 ± 0.05
Flavanols and procyanidins						
Catechin	> 650.00	> 650.00	> 650.00	39.60 ± 1.83	589.24 ± 15.43	0.76 ± 0.18
Epicatechin	605.05 ± 5.71	640.70 ± 9.02	212.87 ± 1.56	< LOQ	30.75 ± 1.38	< LOQ
Epicatechin gallate	311.32 ± 4.19	132.63 ± 1.62	44.43 ± 1.75	< LOQ	< LOQ	< LOQ
Epigallocatechin gallate	2.70 ± 0.21	1.59 ± 0.18	1.07 ± 0.04	< LOQ	< LOQ	< LOQ
Gallo-catechin A (EGCGE)	1.96 ± 0.29	2.17 ± 0.41	1.07 ± 0.17	15.05 ± 0.93	3.17 ± 0.08	< LOQ
Gallo-catechin B (EGCGE)	2.15 ± 0.05	2.29 ± 0.02	1.64 ± 0.06	11.84 ± 0.41	11.98 ± 0.24	< LOQ
Procyanidin A1	0.81 ± 0.23	< LOQ	< LOQ	< LOQ	2.12 ± 0.06	< LOQ
Procyanidin B1	511.58 ± 2.81	363.13 ± 6.93	180.16 ± 3.55	3.38 ± 0.30	102.09 ± 8.23	< LOQ
Procyanidin B2	101.05 ± 5.08	92.80 ± 0.16	22.73 ± 1.74	0.29 ± 0.04	3.02 ± 0.16	< LOQ
Procyanidin B3	58.39 ± 1.27	105.20 ± 3.40	700.84 ± 9.61	17.59 ± 0.67	108.64 ± 1.63	0.11 ± 0.00
PBD 1 (PB1E)	14.41 ± 0.81	26.16 ± 1.14	7.52 ± 0.82	10.09 ± 0.15	37.33 ± 0.80	< LOQ
PBD 2 (PB1E)	47.78 ± 1.76	34.90 ± 2.71	20.85 ± 0.27	< LOQ	21.07 ± 0.38	< LOQ
Procyanidin C1	74.14 ± 2.94	90.73 ± 2.13	19.23 ± 0.53	0.89 ± 0.28	10.51 ± 4.37	0.47 ± 0.06
Flavonols						
Kaempferol	0.61 ± 0.14	0.16 ± 0.02	0.19 ± 0.02	0.16 ± 0.03	0.27 ± 0.02	1.16 ± 0.09
Quercetin	1.97 ± 0.47	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
Taxifolin	0.56 ± 0.01	0.30 ± 0.03	< LOQ	0.12 ± 0.02	0.19 ± 0.02	< LOQ
Naringenin	0.38 ± 0.03	0.10 ± 0.01	< LOQ	< LOQ	0.06 ± 0.01	0.34 ± 0.02
Flavonol glycosides						
Q-TG (RE)	< LOQ	< LOQ	< LOQ	< LOQ	10.99 ± 0.13	< LOQ
Q-NEO (RE)	< LOQ	< LOQ	< LOQ	4.29 ± 0.10	51.19 ± 2.79	0.36 ± 0.04
Q-RUT	< LOQ	< LOQ	< LOQ	0.85 ± 0.28	64.88 ± 1.10	< LOQ
Q-GLUC (RE)	1.68 ± 0.17	1.45 ± 0.17	0.64 ± 0.08	1.19 ± 0.14	240.82 ± 3.19	43.19 ± 2.31
Q-MG (RE)	< LOQ	< LOQ	< LOQ	< LOQ	30.76 ± 1.16	0.37 ± 0.04
Q-SOP (RE)	< LOQ	< LOQ	< LOQ	1.13 ± 0.09	16.00 ± 0.16	0.20 ± 0.02
K-RUT (RE)	< LOQ	< LOQ	< LOQ	2.26 ± 0.22	47.97 ± 0.48	< LOQ
K-GLUC (RE)	0.62 ± 0.24	0.36 ± 0.03	0.56 ± 0.45	0.62 ± 0.13	119.95 ± 1.61	2.80 ± 0.11

Q-MG B (RE)	< LOQ	< LOQ	< LOQ	< LOQ	3.59 ± 0.25	< LOQ
Prenylflavonoids and resins						
Xanthohumol	-	-	-	-	7.09 ± 0.15	2482.70 ± 124.41
Isoxanthohumol	-	-	-	-	4.08 ± 0.13	29.47 ± 0.92
Desmethylxanthohumol (XNE)	-	-	-	-	1.09 ± 0.03	55.54 ± 4.45
6-PN	-	-	-	-	0.10 ± 0.00	2.60 ± 0.09
Cohumulone	-	-	-	-	68.65 ± 0.00	604.09 ± 12.38
Ad/Humulone	-	-	-	-	46.04 ± 0.00	1127.64 ± 12.42
Colupulone	-	-	-	-	< LOQ	68.28 ± 6.20
Ad/Lupulone	-	-	-	-	< LOQ	61.60 ± 5.38
Totals						
Phenolic/chlorogenic acids	88.75 ± 1.5	102.25 ± 2.24	118.13 ± 2.20	9.09 ± 0.92	33.49 ± 0.91	2.45 ± 0.21
Flavanols/procyanidins	> 2331.34 ± 25.01	> 2092.30 ± 27.31	> 1812.41 ± 19.87	98.73 ± 3.27	919.92 ± 32.44	1.34 ± 0.24
Flavonols	3.52 ± 0.65	0.56 ± 0.06	0.19 ± 0.02	0.28 ± 0.05	0.52 ± 0.05	1.5 ± 0.11
Flavonol glycosides	2.30 ± 0.41	1.81 ± 0.20	1.20 ± 0.52	10.36 ± 0.96	586.15 ± 10.87	46.92 ± 2.52
Prenylflavonoids	-	-	-	-	12.36 ± 0.31	2570.31 ± 129.87
Bitter resins	-	-	-	-	114.69 ± 0.00	1861.61 ± 36.38
Total	> 2425.91 ± 27.57	> 2196.92 ± 29.81	> 1931.93 ± 22.61	118.46 ± 5.20	1667.13 ± 44.58	4490.01 ± 169.89

Table 5.4: Phenolic and resin compound concentrations in hop and comparison materials expressed as mg/10 g.
Data represent the mean ± standard deviation of triplicate analytical measurements (n=3).
EGCGE: epigallocatechin gallate equivalent, **RE**: rutin equivalent, **CQAE**: chlorogenic acid equivalent, **3,4-DCQAE**: 3,4-Dicaffeoylquinic acid equivalent, **PB1E**; procyanidin B1 equivalent, **XNE**: xanthohumol equivalent.

ProAntho was predominantly made up of non-galloylated catechins, B-type procyanidins and flavonol 3-O-glycosides, with hydroxycinnamic and chlorogenic acids present at lower concentrations (Figure 5.10, Table 5.4). This is in agreement with Luzak et al. (2016) who found that these groups are the most abundant hydrophilic phenolics in purified extracts from hop CO_2R. ProXantho was made up of prenylflavonoids and bitter resins with xanthohumol the most abundant compound. Phenolic composition of hop fractions generally reflected that of the CO_2R-HERK crude extract detailed in Chapter 4, but with compounds separated based on hydrophobicity with the precipitation of prenylflavonoids and bitter resins during ethanol removal.

LC-MS total Ion Chromatograms (TICs) of ProAntho and ProXantho are shown in in Figure 5.11. One of the most abundant peaks in the ProAntho extract appears at retention time (RT) 11.70, with an *m/z* of 357.12. This peak, previously identified as particularly abundant in the CO_2R-HERK representative extract, was tentatively suggested to be co-multifidol glucoside, a phloroglucinol derivative commonly found at high levels in Herkules hop cones (Schmidt and Biendl, 2023b). Although this peak was not reported on in the present study due to low match scores from SIRIUS and CSIFingerID, its transfer to the ProAntho fraction through PAD950 adsorption provides further evidence of a phenolic structure. Due to its high abundance, future work should adopt structural determination techniques such as nuclear magnetic resonance to confirm this as reference standards are not currently commercially available.

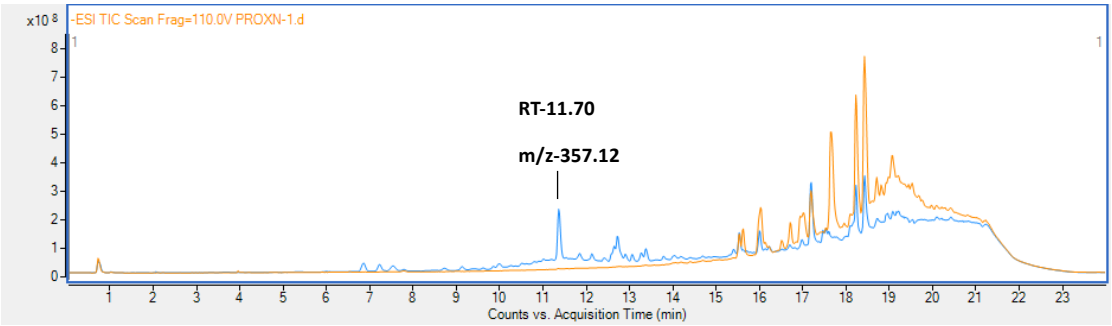


Figure 5.11: LC-MS total ion chromatograms (TIC's) of ProAntho (blue) and ProXantho (yellow) extracts presented in overlay mode.

In the HCA (Figure 5.10), Grape OPC, Vitisol and Pineol were most similar to one another in phenolic composition, with the highest levels of B-type procyanidins and catechins among all the materials analysed. The catechin abundance for Grape OPC, Vitisol and Pineol exceeded the highest concentration of the standard curve, which was 600 mg/10 g for these extracts. ProAntho contained lower concentrations of B-type procyanidin dimer and trimers compared to Grape OPC, Pineol and Vitisol, but a higher PAC as determined by the acid butanol assay (Figures 5.8 and 5.9). This indicates that hop proanthocyanidins have a higher degree of polymerisation compared to those in grape and bark extracts, which cannot be detected with

the employed LC-MS method (m/z range 50-1700 kDa). CyanthOx 50 had particularly low concentrations for all compounds other than gallo-catechin A and B (MS2), which is unexpected given its previous identification as a source of catechins and B-type procyanidins (Zhu et al., 2021) and PAC of 188.33 mg PB3E/g (Figure 5.9). This discrepancy may be due to CyanthOx 50 containing different procyanidins not analysed in this study. Comparison extracts were not included in structural annotation analysis outlined in 2.2.6.2 which represents a limitation of this study.

To evaluate relative transfer rates between phenolics and resins from the crude E50 extract (detailed in Chapter 4) to the purified hop fractions, ratios were calculated using the formula: Purified fraction IS normalised abundance / Crude extract IS normalised abundance. These ratios are presented in Figure 5.12 as a heat map, with variables ordered according to AHC analysis. Variables with IS normalised abundance < 0.1 were omitted from the analysis.

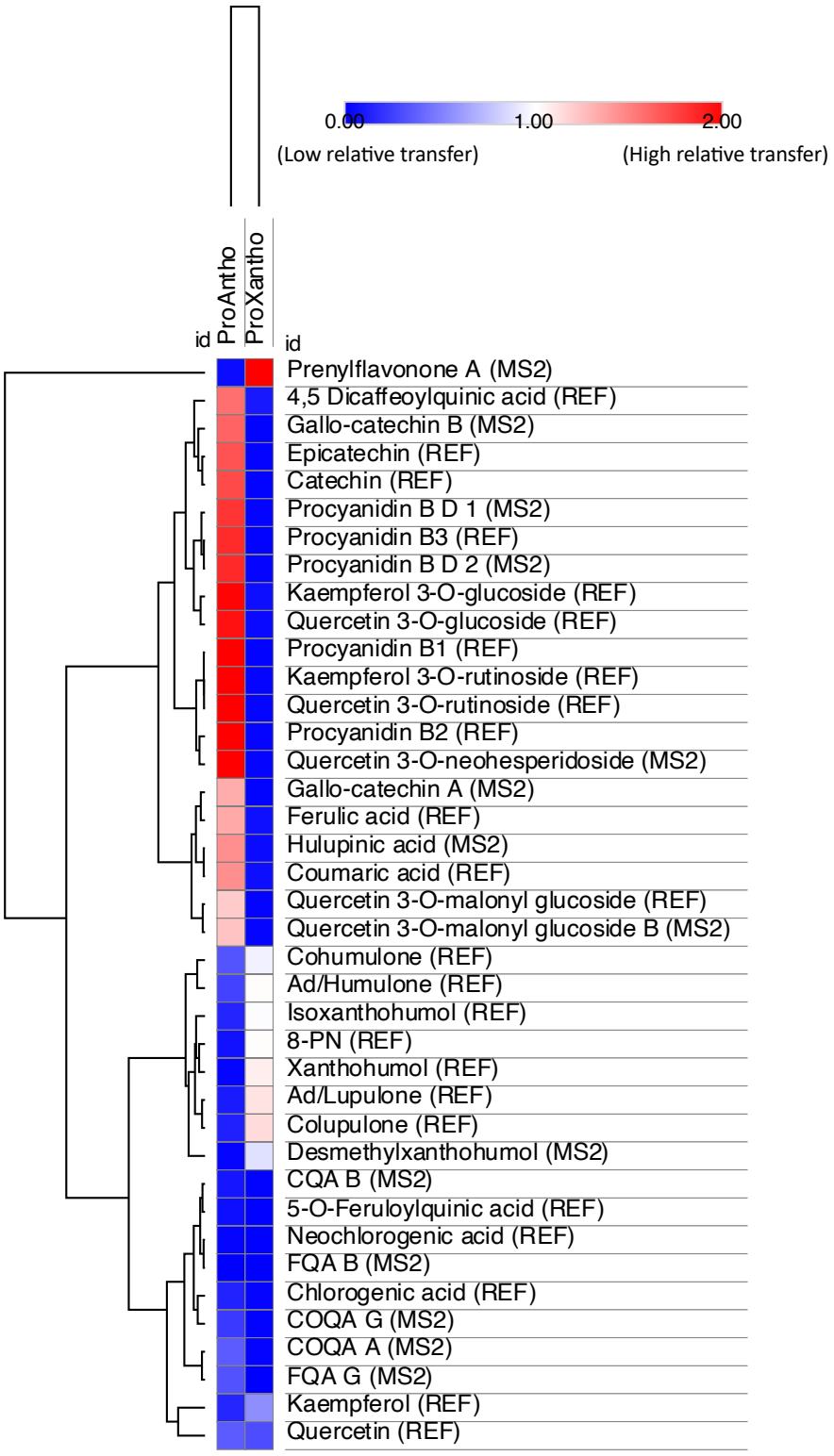


Figure 5.12: Relative transfer rates between phenolics and resins from crude E50 extract to purified hop fractions. Ratios calculated using formula: Purified IS normalised abundance / Crude E50 extract IS normalised abundance.

REF: Compounds identified with reference standards, **MS2**: Compounds identified with mass fragmentation patterns.

B type procyanidins, catechins and flavonol 3-O-glycosides exhibited the highest transfer rates from the crude E50 extract to ProAntho, whilst malonylated quercetin 3-O-glycosides had the lowest transfer rates among all glycosides. This could be related to the malonyl group adding additional polarity to the compounds making them more hydrophilic and reducing their interaction with the hydrophobic PAD950 resin, thereby leading to lower retention and transfer rates. All chlorogenic acids, apart from 4,5-dicaffeoylquinic acid, exhibited poor transfer rates to both fractions. Macroporous resins have been shown to vary in their affinity for chlorogenic acids depending on their chemical nature and polarity (Jiang et al., 2020). This suggests that PAD950 resin is not suitable for phenolic purification for materials where chlorogenic acids and malonyl glycosides are present at high levels, such as S. Passion and S. Aroma hop leaves, as highlighted in Chapter 4. Further research on purifying chlorogenic acids from hop co-products should evaluate different resins as well as adsorption and desorption conditions to optimise their transfer to the purified phenolic fraction.

5.4.5-Antioxidant Activity of hop fractions and commercial extracts

ProAntho, Proxantho and selected comparison extracts and phenolic standards were analysed for antioxidant activity using the DPPH, FRAP and ORAC assays. Representative standards were included from the dominant phenolic sub-groups in the extracts including prenylflavonols (xanthohumol), procyanidins (procyanidin B3), quercetin glycosides (quercetin 3-O-glucoside) and kaempferol glycosides (kaempferol 3-O-glucoside). Antioxidant activity was expressed as Trolox equivalents (mg TE/g), and data are presented in Figure 5.13.

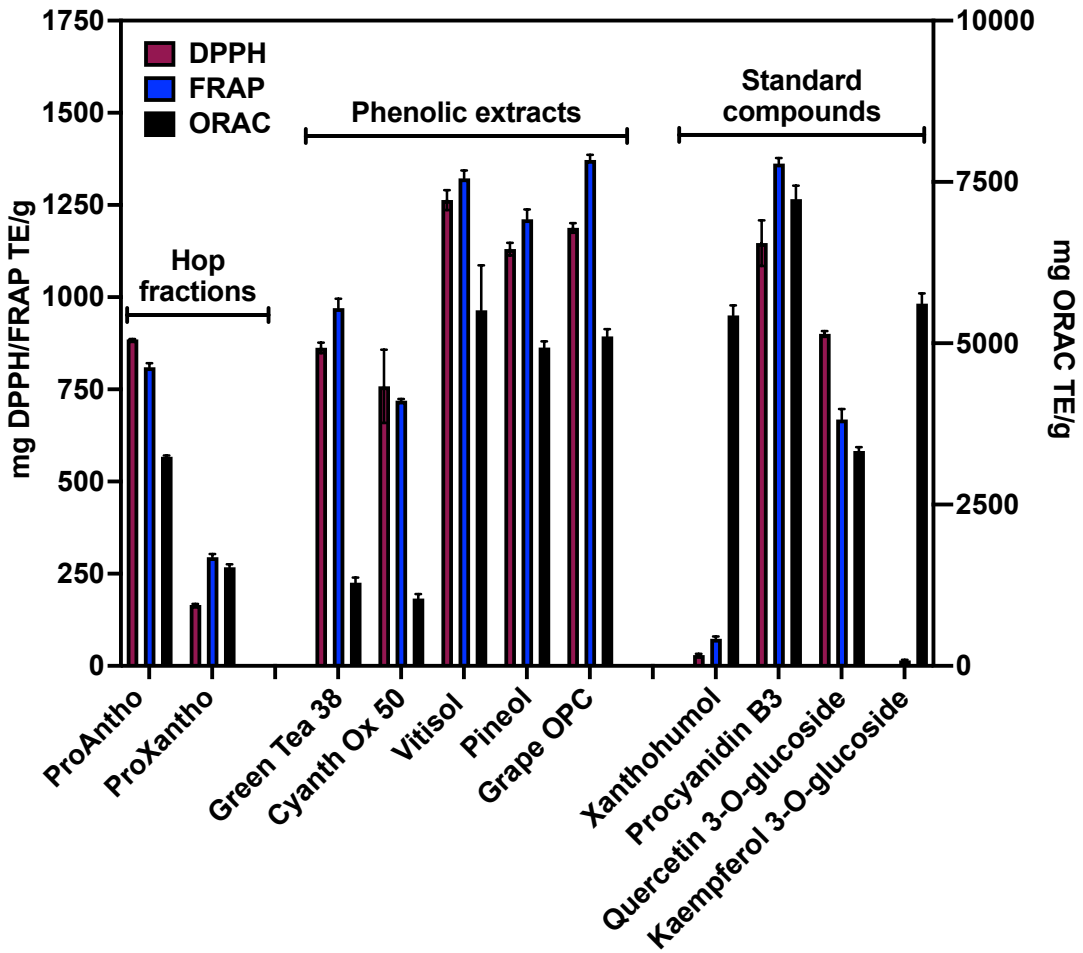


Figure 5.13: DPPH, FRAP and ORAC antioxidant activity of hop fractions, commercial phenolic extracts and standard compounds. Antioxidant activity expressed as Trolox equivalents (mg TE/g).

Data represent the mean ± standard deviation of triplicate analytical measurements (n=3).

ProAntho exhibited higher antioxidant activity across all assays compared to ProXantho. In comparison to CO_2R-HERK antioxidant activity (detailed in Chapter 4), ProAntho exhibited fold increases in DPPH, FRAP and ORAC activities of 22.3, 19.1 and 11.1 respectively, whereas ProXantho fold increases were lower, at 4.2, 7.0 and 5.22 respectively. Of all extracts analysed, ProXantho had the lowest FRAP and DPPH activity relative to ORAC activity. This is likely related to the high content of prenylflavonoids in particular xanthohumol, which exhibits negligible ET based antioxidant activity (Santos and Silva, 2020), as shown by analysis of the pure compound (Figure 5.13). ProAntho antioxidant activities were generally comparable to those of Green Tea 38 and Cyanth Ox 50, but lower relative to Vitisol, Pineol and Grape OPC.

The lower antioxidant activity of ProAntho compared to the Berkhem grape and bark proanthocyanidin extracts may be attributed to differences in phenolic selectivity during the purification process, and/or differences in phenolic composition. Selectivity differences could influence phenolic purity whilst differences in phenolic composition may affect antioxidant properties, which are known to vary depending on phenolic structure (Vuolo et al., 2019), as highlighted by the pure compound data in Figure 5.13. ProAntho was predominantly made up of high molecular weight B-type proanthocyanidins as well as quercetin and kaempferol 3-O-glycosides whilst Berkehem extracts were made up of catechins and oligomeric proanthocyanidins. There are differing reports on the impact of degree of polymerisation on the antioxidant properties of proanthocyanidins, however generally oligomeric PACs in particular dimers and trimers have been shown to be more effective antioxidants compared to polymeric PACs (Plumb et al., 1998, Arimboor and Arumughan, 2012, Navarro et al., 2017). Zhu et al. (2019) found that polymeric PAC's from *L. gmelinii* bark had around 50% lower DPPH radical scavenging activity compared to oligomeric PAC's and that depolymerisation by catalytic hydrogenolysis was effective for improving the antioxidant activity of polymeric PAC's.

Similarly, flavonol 3-O-glycosides have been shown to have lower antioxidant activities compared to B-type procyanidins and their respective aglycones, depending on the flavonol and position of glycosylation (Vuolo et al., 2019). This can be observed in Figure 5.13 where quercetin and kaempferol 3-O-glycoside, the most abundant flavonol glycosides in ProAntho exhibited lower activity than procyanidin B3 for all antioxidant assays. It was discussed in 5.4.4 that co-multifidol glucoside may be a major phenolic component of ProAntho. Spreng and Hofmann (2018) identified co-multifidol glucoside as a major antioxidant in beer via an activity guided approach. However, ORAC analysis of the standard by Spreng and Hofmann (2018) revealed lower antioxidant activity on a molar basis (TE-2.16) compared to flavonol glycosides such as quercetin-3-O-glucoside (TE-3.94).

Aside from differences in phenolic composition, selectivity of the purification techniques could also play a role in antioxidant properties of the resulting extracts. Cifuentes-Cabezas et al. (2022) evaluated PAD950 resin for phenolic purification and found higher levels of adsorbed sugars (19.28%) compared to other resins such as MN200 and MN202. This indicates that PAD950 may be less suited to phenolic purification due to lower specificity, with sugars from the crude aqueous hop extract competing for resin sites, thereby resulting in a lower antioxidant activity in the extract. This aligns with the lower TPC of ProAntho compared to Berkhem extracts (Figures 5.8 and 5.9).

The total phenolic and bitter resin content of ProXantho was 44.84% (Table 5.4) however it is unclear what makes up the remaining 55.16%. Grudniewska and Pastyrczyk (2023) investigated the isolation of xanthohumol via precipitation from deep eutectic solvent extracts from CO_2R-Magnum and found along with xanthohumol, precipitates had a high protein content (40-84%). This suggests that the remaining 55.16% of ProXantho may also be made up of protein thereby reducing phenolic purity and antioxidant activity. The purification technique used to produce the Berkhem extracts is not stated, however the lower antioxidant activity of hop fractions compared to these extracts seems in part related to the presence of impurities due to the lower selectivity of PAD950 and precipitation compared to other SPE resins for phenolics.

Chapter 6-Valorisation of hop leaves for their bioactive compounds: Identification and quantification of phenolics across different varieties, crop years and stages of development.

6.1-Introduction

Chapter 5 focused primarily on phenolics from CO_2 extract residue cv. Herkules (CO_2R-HERK) due to its high prenylflavonoid content and high material availability to industry, as Herkules is currently the variety of choice for CO_2 extraction. However, in Chapter 4 it was highlighted that hop leaf extracts exhibited comparable phenolic contents and antioxidant activities to hop processing residues and were a richer source of flavonol 3-O-glycosides and chlorogenic acids. Whilst hop cones have been well characterised for their phenolic content (Schmidt and Biendl, 2023b), hop leaves have received less attention. The current literature on the phenolic content and antioxidant activity of leaf extracts is discussed in section 1.4 which highlights that quantitative data on the most abundant phenolics in hop leaves is currently limited. This is important in determining appropriate extraction and purification processes as well as informing what the highest value application should be.

In Chapter 4, flavonol glycosides in South African hop leaf extracts were semi-quantified using rutin equivalents, however authentic compound standards are required for accurate quantitative analysis. Additionally, only two South African leaf varieties were evaluated, sourced from a region with significantly lower acreage (408 ha, 2023 crop) compared to major hop growing regions such as Washington, USA (15,723 ha, 2023 crop) (Barth-Haas-Group, 2023). In this study the objective was to assess the phenolic composition of hop leaves for three commercially significant hop varieties grown in the Yakima valley of Washington over two harvest years at different stages of development post-flowering. The overall aims were to evaluate the feasibility of hop leaf valorization for phenolic content and to gain new insights into their biochemistry.

6.2-Materials

6.2.1-Chemicals

Formic acid (LC-MS grade) was obtained from Fisher Scientific (Loughborough, UK). All other chemicals and reference standards were obtained according to Chapter 2.

6.2.2-Hop materials

Hop leaf material was obtained for three varieties (Calypso-CAL, Cascade-CAS and Contessa-CON), across three stages of development (Flower-FL, Middle-MID and Harvest-HV) for two crop years (2021 and 2022). Young (Y) and old (O) leaves for Calypso were collected based on size for both crop years. Respective cones (CONE) were collected for all varieties for crop year 2021. Hop leaf and cone samples were collected from a single commercial irrigated hop field on 'Roza Ranch' located at latitude: 46.29164, longitude: -119.78733 within the Washington Yakima Valley. All varieties were rootstock planted in 2020 and cultivated using organic practices as specified by the Washington State Department of Agriculture (WSDA) organic program accredited by the United States Department of Agriculture (USDA).

Leaf collection

Each leaf sample consisted of 10 kg of wet leaves collected by hand within the field by removing leaves from petioles. Any leaves with significant visual damage from biotic or abiotic stress were excluded from sampling. Leaves from stages FL, MID, HV and O were collected from side arms branching from nodes 1.2 to 1.5 m above the ground. Stage O leaf samples were comprised of recalcitrant leaves at least two weeks past full expansion collected from petiole positions closer to the main bine on side arms. Stage Y leaf samples were collected by stripping all leaves from fresh shoots newly emerged from the crown and cut off at ground level. Stage FL, MID and HV leaves were comprised of older, recalcitrant leaves and new, fully expanded leaves. A visualisation of the different developmental stages of hop leaves sourced for this study is presented in Figure 6.1. Mesh nylon bags were used to

collect leaves within the field. All leaf samples were collected post chemical pruning. Stage FL leaf samples were collected at time of bloom emergence for each variety which varied depending on biotic and abiotic factors. Stage Y and O leaf samples were collected whilst bloom was still present. Stage MID leaf collection corresponded to late bloom and early cone development. Stage HV collection was performed two days prior to harvest of cones. Dates of sample collection for leaves and cones are provided in Appendix 6, Figure 1.

Figure 6.1: Overview of the developmental stages of hop leaves analysed in this study.

Initial wet leaf dry matter ranged from 30-40%. The nylon bags were placed inside an electric mini hop kiln and dried at 63 °C to a target dry matter of range of 85-92.5%. Total kilning time ranged from 210-300 min. Post kilning all samples were transferred to laminate foil bags which were then flushed with nitrogen and sealed. After packaging samples were stored at 5 °C until shipment for analysis.

Climate data

Temperature and UV radiation data for 2021 and 2022 were obtained from Washington State University (WSU) AgWeatherNet weather station located at Lat: 46.2950332 Long: -

119.7298231. This is 5 km east of the hop farm located in the same southeast end of the Yakima valley and should provide climate data representative of the hop farm conditions. Average 7-day temperature and solar radiation data from the WSU weather station is presented and described in Appendix 6, Figure 1.

6.3-Methods

6.3.1-Extraction of phenolics from hop materials

Hop materials were extracted using 50% aqueous ethanol (v/v) (E50) using methodology outlined in 2.2.1. Extractions were performed in triplicate per material.

6.3.2-Determination of phenolic content using spectrophotometric assays

Total phenolic content (TPC) and proanthocyanidin content (PAC) of extracts was determined using methodology outlined in 2.3. For TPC analysis hop extracts were diluted E50 analyzed at 10mg/ml in triplicate and expressed as caffeic acid equivalents (CAE). For proanthocyanidin content extracts were analyzed at 100 mg/ml in triplicate and expressed as procyanidin B3 equivalents (PB3E).

6.3.3- Analysis of xanthohumol and bitter acids (HPLC-UV-DAD)

HPLC-DAD was used for the quantitation of xanthohumol, α and β-acids in cones and α and β-acids in leaves using methodology outlined in 2.4. Extracts were analysed at 100 mg/ml in triplicate in a randomised order and expressed as w/w DM.

6.3.4-Screening and quantitative analysis of phenolics (LC-ESI-qTOF-MS/MS)

Phenolic screening and quantitative analysis were performed using LC-ESI-qTOF-MS/MS following methodology outlined in 2.4. Internal standard (IS) solution was made up of all 18 candidate compounds (outlined in Table 2.3) at 25 µg/ml 1% (v/v) formic acid E50. Hop extracts were syringe filtered at 0.22 µm, diluted with E50 and analysed at 9.09 mg/ml with IS candidates at a final concentration of 2.5 µg/ml. External standards were dissolved in E50,

diluted across 4 orders of magnitude to 16 concentrations (concentrations ranges provided in Appendix 6, Table 1) and analysed in quadruplicate. Hop extracts were analysed in duplicate per extraction (six replicates per condition) and injected (1 µl) in a randomised order. A QC sample comprising equal parts of each extract was analysed after every 12 sample injections to check system performance.

Fine quantitation for selected polyphenols was performed using standard curves outlined in Appendix 6, Table 1 and results were expressed as w/w DM. For sample solutions where kaempferol 3-O-rutinoside and quercetin 3-O-glucoside concentrations were between 24 and 104 µg/ml, a quadratic polynomial calibration was fitted as shown in Appendix 6, Figure 2. For quantitation, peak areas for each compound were normalised to an optimum internal standard, which was chosen based on structural similarity to target compound and the ability to reduce standard deviation of target compounds in both QC injections and each standard curve. Relative quantitation for phenolic screening was performed by normalising peak areas to hesperidin and DM thereafter.

6.4-Results and Discussion

6.4.1-Moisture content of leaf materials

The moisture contents of hop leaves varied considerably for the 2021 crop year samples (5.76-23.06%) but was more stable for 2022 (7.99-10.96%). Drying is an essential pre-treatment for phenolic extraction, as it facilitates cell wall breakage and the formation of cavities, thereby enabling cellular components to be extracted (Drosou et al., 2015). To assess the impact of moisture content on the phenolic content of hop leaves, correlation analysis was performed between TPC and moisture content for all leaf samples which is presented in Figure 6.2.

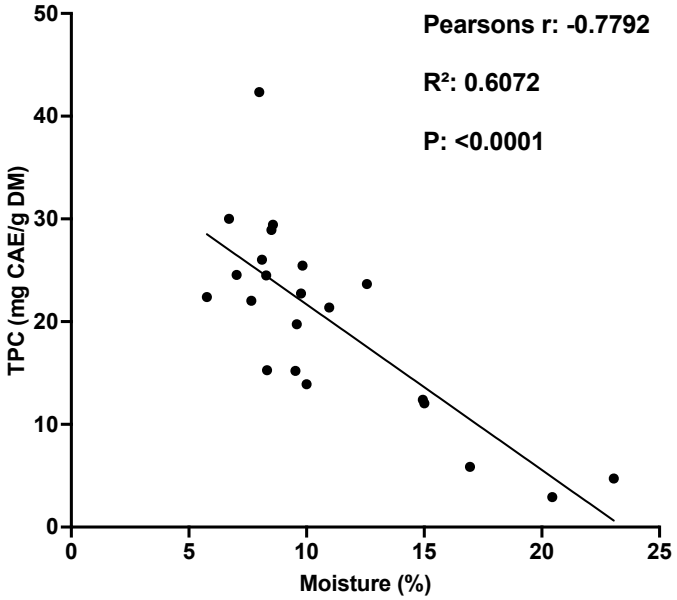


Figure 6.2: Pearson's correlation analysis between Total Phenol Content (TPC) and moisture content of hop leaf samples for both crop years.

TPC data represents the mean of triplicate extractions (n=3).

CAE: Caffeic acid equivalents.

A statistically significant inverse correlation was observed between the moisture content and TPC of hop leaves (P <0.0001). This relationship was most pronounced when the moisture content exceeded 15% (Figure 6.2). Therefore, samples with moisture levels above 15% were removed from further analysis to limit the confounding effects of moisture on the factors under evaluation in this study. Macchioni et al. (2021) highlighted that extracts of oven-dried hop leaves (such as those evaluated in this study) had lower antioxidant activity compared to their respective freeze-dried leaf extracts, indicating that phenolics are degraded during oven drying. Freeze drying generally results in lower variability in the moisture content of leaves compared to oven drying where differences in heat distribution can lead to uneven moisture removal (Abascal et al., 2005). This indicates freeze-dried hop leaves could be more suited for evaluating the impact of variety, stage of development, crop year and environmental factors on hop leaf phenolic composition. However, freeze-drying is generally not considered economically viable for agri-food residues for development of commercial phenolic extracts

(Berenguer et al., 2022), therefore oven-drying was selected in this study. The commercial viability of drying hop leaves for phenolic extraction is discussed further in 6.4.6.

6.4.2-Total phenolic and proanthocyanidin contents of hop leaves and cones

Figure 6.3 presents the TPC and PAC of hop leaf and cone extracts. The TPC and PAC of all leaf samples were substantially lower than those of their respective cones. This is in agreement with most prior research comparing hop leaves and cones; for example, it has been reported that leaves had a lower PAC and a 3-30 fold lower TPC (Abram et al., 2015). TPC analysis indicates cones had a higher phenolic concentration, however this result could also be due to greater reduction activity of cone phenolics in the assay (Platzer et al., 2021). Interestingly, all Yakima grown leaf samples analysed in this study had significantly lower TPC compared to the South African leaf extracts evaluated in Chapter 4. It is not clear if this is attributable to variety, cultivation location or other factors however it highlights the variability of the phenolic content of hop leaves.

Accumulation patterns over stage of leaf development were very similar for TPC and PAC across all samples. However, accumulation patterns differed depending on variety and were not consistent between crop years. TPC and PAC were more variable between stage of leaf development in 2021, whereas levels were more consistent across different growth stages for all varieties in 2022. In the one direct comparison made between young and old leaves sampled on the same day (Calypso 2022), young leaves contained significantly higher amounts of TPC and PAC than old leaves ($P<0.05$). Previous studies have shown significant differences in the phenolic content of leaves over maturation, and young leaves have been found to be a richer source compared to old leaves in other plants such as green tea, possibly due to higher metabolic activity (Lin et al., 1996).

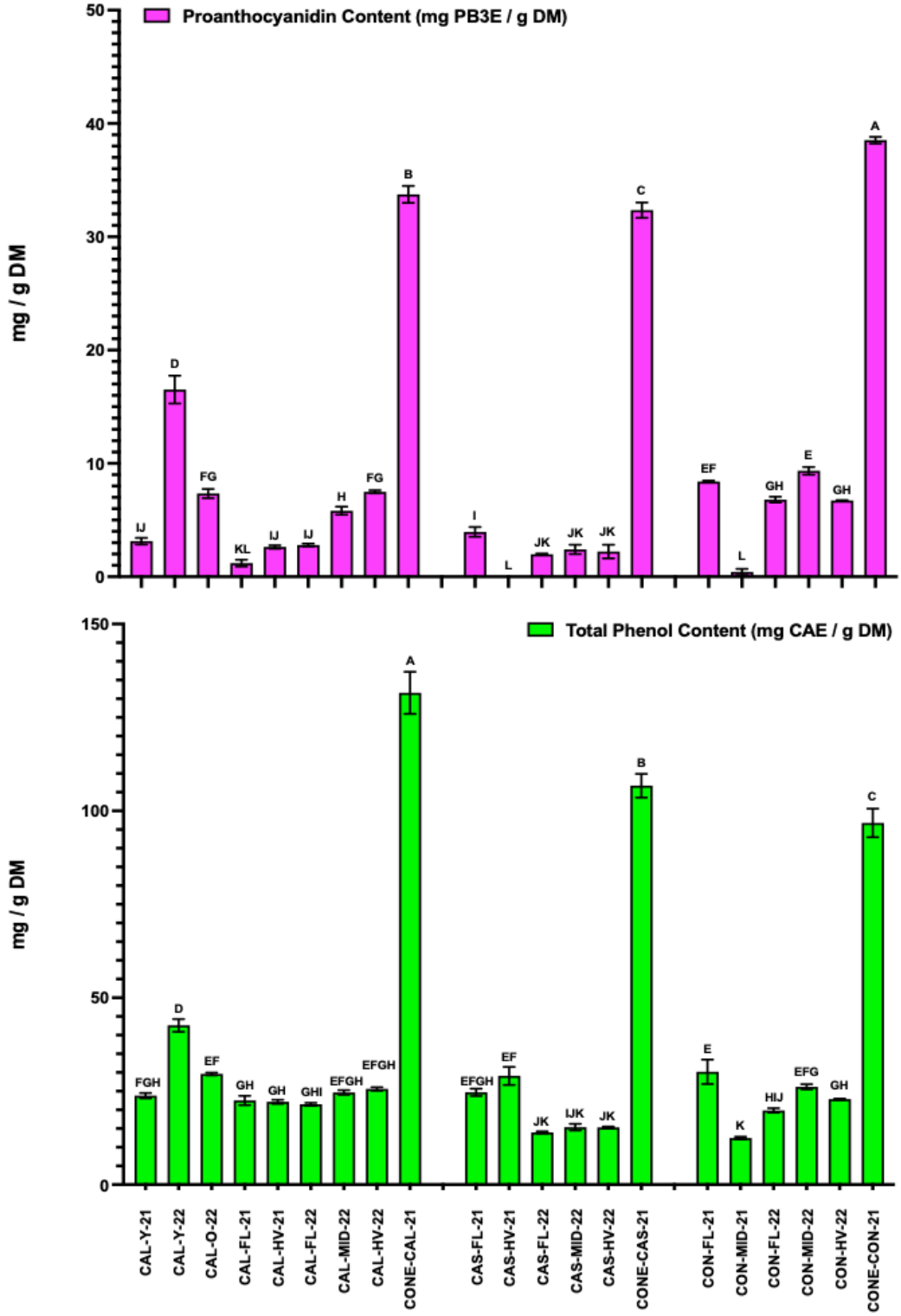


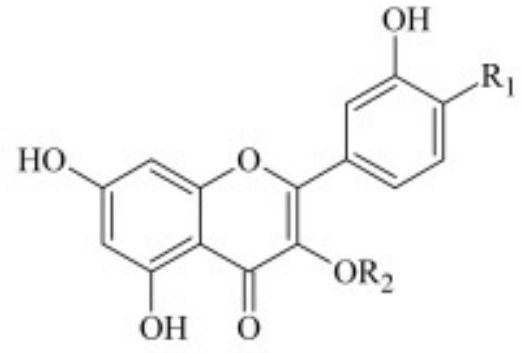
Figure 6.3: Total Phenolic Content (TPC) and Proanthocyanidin Content (PAC) of hop leaf and cone extracts.

Data represent the mean ± standard deviation of triplicate extractions (n=3). Letters represent ANOVA post-hoc groupings ($P < 0.05$).

CAL-Calypso. **CAS**-Cascade. **CON**-Contessa. **FL**-Flower stage, **MID**-Middle stage, **HV**-Harvest stage. **21**-2021 crop year. **22**-2022 crop year. CONE-Hop cones. **CAE**: caffeic acid equivalents. **PB3E**: procyanidin B3 equivalents.

6.4.3-Qualitative and quantitative analysis of phenolics

Structural annotation using MS/MS in silico fragmentation was carried out on the QC extract as outlined in 2.2.6.2.1.2. Analysis identified additional kaempferol and quercetin 3-O-glycosides and chlorogenic acids to those identified in the other three representative extracts, with all features reported on in this study. The MS/MS fragmentation, SIRIUS and CSI scores of these features are presented in Table 2.4. General structures for the different phenolic classes are presented in Figure 2.3 and 2.4. The structures of the most abundant flavonol glycosides identified in this study are presented in Figure 6.4.



Compound	R_1	R_2
Kaempferol 3-O-rutinoside	H	rutinose
Kaempferol 3-O-(6''-O-malonylglucoside)	H	6''-O-malonylglucose
Kaempferol 3-O-glucoside	H	glucose
Kaempferol 3-O-galactoside	H	galactose
Clitorin	H	rhamnose-rutinose
Quercetin 3-O-rutinoside	OH	rutinose
Quercetin 3-O-(6''-O-malonylglucoside)	OH	6''-O-malonylglucose
Quercetin 3-O-glucoside	OH	glucose
Manghaslin	OH	rhamnose-rutinose

Figure 6.4: Structures of the most abundant flavonol glycosides identified in the hop leaf extracts (Karabin et al., 2015).

Figure 6.5 presents relative quantitation for all phenolics identified as a normalised heat map with samples and phenolic variables ordered according to AHC analysis. The key phenolics were quantified to assess valorisation potential of hop leaves and to compare with published literature on other agri-food co-products being assessed for phenolic content. Standards for quantitation were chosen based on preliminary analysis of the most abundant phenolics in

the QC sample, literature sources (da Silva et al., 2021, Morcol et al., 2021) and commercial availability. Abundance of kaempferol 3-O-(6''-O-malonylglucoside) (K-MG), clitorin, desmethylxanthohumol and coumaroylquinic acid A (COQA A) identified with MS2 was very high in the QC sample, however standards were not available at the time of analysis for quantitation. Quantitative data are presented in Tables 6.1-6.4 for flavonols and their glycosides, chlorogenic and phenolic acids, flavanols and procyanidins as well as prenylflavonols and bitter resins respectively. To our knowledge this is the first time the dominant phenolics have been quantified in hop leaves. Although hop cone phenolic profiles have been well characterised, in this study they serve as a useful reference material for leaf assessment.

The most significant difference in phenolic profile of materials analysed was between hop leaves and cones as shown by the cluster analysis in Figure 6.5. Cone phenolic abundance generally followed the order: prenylflavonoids > flavanols and procyanidins > flavonol glycosides > chlorogenic acids > phenolic acids, whilst leaves followed the order: flavonol glycosides, chlorogenic acids > flavanols and procyanidins > prenylflavonoids > phenolic acids.

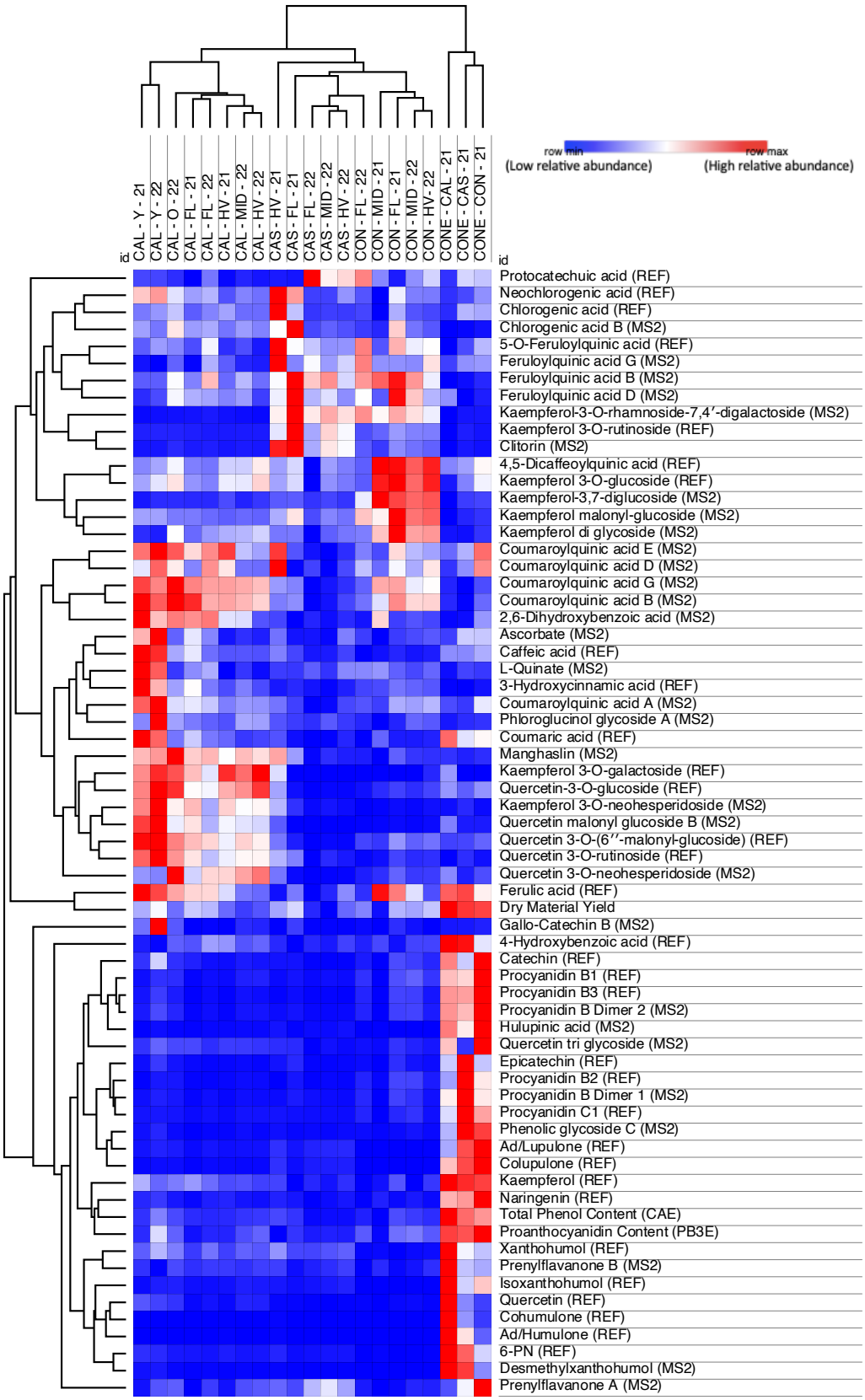


Figure 6.5: Normalised heat map of phenolic variables for hop leaves and cones with samples and variables ordered according to AHC analysis (Euclidean distance).

CAL-Calypso. **CAS**-Cascade. **CON**-Contessa. **FL**-Flower stage, **MID**-Middle stage, **HV**-Harvest stage. **21**-2021 crop year. **22**-2022 crop year. **CONE**-Hop cone.

Flavonol glycosides

The dominant flavonol glycosides in hop leaves and cones were mono, di and tri glycosylated forms of quercetin and kaempferol. These were all 3-O-glycosides which are generally more common than 7', 3' and 4' glycosides in hops (Dušek et al., 2021) and other plant species (Belitz et al., 2008). Flavonol glycosides were quantitatively the main phenolic components of hop leaf samples for all stages of development, crop years and varieties and were found at substantially higher levels (184.8-843.4 mg/100 g DM) than in the respective cones (107.9-170.3 mg DM/100 g DM). They were also generally found at higher levels compared to the South African leaf extracts evaluated in Chapter 4, which semi-quantified flavonol glycosides as rutin equivalents. This was in contrast to the lower TPC of leaf extracts from Yakima compared to those from South Africa.

Generally, Calypso and Cascade leaves contained greater amounts of quantified glycosides than Contessa leaves (Table 6.1, Figure 6.5). In contrast, the aglycones quercetin and kaempferol were present at higher concentrations in hop cones compared to leaves. However, concentrations were still significantly lower than for their respective glycosides (Table 6.1). Flavonol glycosides are generally found in higher levels than their respective aglycones in plant materials including hop cones and shoots, however there is limited literature on their prevalence in hop leaf material (Maietti et al., 2017). Structurally diverse glycosylated and malonylated flavonols are prevalent in the leaves of many different plant species (Sugiyama et al., 2013).

It is apparent from Figure 6.5 that variety had a strong impact on the accumulation of flavonol glycosides. For example, Calypso leaves had the highest levels of all quercetin glycosides, K-GALAC and K-NEO but lower levels of kaempferol di- and tri- glycosides. Young Calypso leaves from both crop years generally contained the highest levels of glycosides. Contessa and Cascade glycoside profiles were more similar to one another. In Cascade leaves kaempferol

di- and tri- glycosides predominated. K-RUT was the main polyphenol in Cascade leaves with concentrations ranging from 247.7-744.2 mg/100 g DM. Contessa leaf flavonol glycosides profile was characterised by higher levels of K-MG, K-GLUC and K-3,7-DIGLUC, with lower levels of K-RUT, clitorin and K-3-R-7,4-DIGALAC (Table 6.1, Figure 6.5).

Table 6.1: Flavonol and flavonol glycosides contents in hop leaves (LF) and cones (CONE) (mg/100 g DM).

	Q	Q-GLUC	Q-M GLUC	Q-RUT	K	K-GLUC	K-GALAC	K-RUT
LF-CAL-Y-21	1.57 ± 0.05 c	112.60 ± 2.13 c	228.45 ± 11.27 a	140.06 ± 2.13 b	0.32 ± 0.03 c	20.21 ± 0.29 g	8.33 ± 0.26 cd	50.03 ± 1.45 j
LF-CAL-Y-22	1.24 ± 0.49 cd	151.29 ± 3.59 a	232.27 ± 1.30 a	169.86 ± 3.70 a	0.34 ± 0.03 c	26.05 ± 0.64 ef	10.55 ± 0.62 ab	56.24 ± 1.60 j
LF-CAL-O-22	0.97 ± 0.08 de	137.64 ± 2.90 b	174.28 ± 3.63 b	124.88 ± 3.76 c	0.27 ± 0.02 cd	30.98 ± 1.25 d	9.63 ± 0.48 a-c	54.18 ± 1.70 j
LF-CAL-FL-21	0.22 ± 0.06 fg	76.69 ± 0.82 e	147.94 ± 2.17 c	100.26 ± 1.25 d	0.32 ± 0.03 c	18.76 ± 0.47 gh	7.19 ± 0.90 d	49.13 ± 0.64 j
LF-CAL-HV-21	0.22 ± 0.06 fg	99.98 ± 1.47 d	110.93 ± 3.02 f	87.32 ± 2.17 f	0.18 ± 0.03 def	26.81 ± 0.48 ef	10.50 ± 0.48 ab	40.74 ± 0.70 jk
LF-CAL-FL-22	0.40 ± 0.02 fg	72.99 ± 0.89 e	136.86 ± 6.96 d	72.58 ± 1.57 g	0.19 ± 0.01 de	17.07 ± 0.15 hi	5.13 ± 0.43 e	40.71 ± 0.80 jk
LF-CAL-MID-22	0.69 ± 0.02 ef	108.87 ± 2.71 d	138.65 ± 2.95 cd	94.61 ± 2.36 e	0.20 ± 0.01 de	26.40 ± 0.64 ef	9.01 ± 1.03 b-d	43.28 ± 0.68 jk
LF-CAL-HV-22	0.63 ± 0.04 e-g	135.24 ± 5.59 b	124.37 ± 3.10 e	95.46 ± 3.20 de	0.17 ± 0.02 def	32.51 ± 1.30 d	11.47 ± 0.91 a	42.70 ± 1.75 jk
CONE-CAL-21	8.94 ± 0.61 a	44.83 ± 1.68 f	32.60 ± 0.73 j	14.39 ± 0.59 m	0.92 ± 0.05 a	7.54 ± 0.33 k	3.43 ± 0.32 f	5.10 ± 0.16 l
LF-CAS-FL-21	0.17 ± 0.00 fg	4.72 ± 0.11 k	16.12 ± 0.76 k	53.24 ± 0.34 h	< LOQ h	25.04 ± 0.35 f	< LOQ g	744.24 ± 17.18 a
LF-CAS-HV-21	0.19 ± 0.05 fg	49.02 ± 1.44 f	56.61 ± 1.26 h	69.05 ± 2.05 g	< LOQ h	20.15 ± 0.74 g	4.96 ± 0.33 e	336.81 ± 7.06 d
LF-CAS-FL-22	0.13 ± 0.03 g	0.48 ± 0.01 k	3.33 ± 0.17 l	14.23 ± 0.21 m	0.07 ± 0.01 gh	5.36 ± 0.09 l	< LOQ g	247.65 ± 3.94 e
LF-CAS-MID-22	0.09 ± 0.03 g	2.34 ± 0.10 k	6.42 ± 0.46 kl	26.79 ± 0.39 jk	< LOQ h	18.54 ± 0.24 gh	< LOQ g	437.76 ± 8.14 b
LF-CAS-HV-22	0.14 ± 0.04 fg	1.91 ± 0.07 k	5.37 ± 0.07 l	21.67 ± 0.14 kl	0.08 ± 0.01 f-h	16.34 ± 0.21 i	< LOQ g	364.13 ± 3.92 c
CONE-CAS-21	2.45 ± 0.10 b	28.27 ± 0.17 g	34.42 ± 0.85 j	28.07 ± 0.68 j	0.85 ± 0.02 ab	17.14 ± 0.65 hi	< LOQ g	47.32 ± 1.57 jk
LF-CON-FL-21	0.21 ± 0.04 fg	19.50 ± 0.68 hi	70.02 ± 1.04 g	34.67 ± 0.50 i	0.15 ± 0.02 e-g	51.56 ± 1.44 a	< LOQ g	228.62 ± 5.02 f
LF-CON-MID-21	0.09 ± 0.05 g	13.17 ± 0.10 j	27.35 ± 0.83 j	18.54 ± 0.19 lm	< LOQ h	49.23 ± 0.78 b	< LOQ g	150.15 ± 2.36 h
LF-CON-FL-22	0.15 ± 0.04 fg	4.81 ± 0.11 k	33.85 ± 0.92 j	15.80 ± 0.39 m	< LOQ h	13.64 ± 0.35 j	< LOQ g	116.71 ± 3.94 i
LF-CON-MID-22	0.24 ± 0.04 fg	13.64 ± 0.45 ij	46.26 ± 0.74 i	26.98 ± 1.25 jk	< LOQ h	42.10 ± 0.79 c	< LOQ g	191.86 ± 5.17 g
LF-CON-HV-22	0.15 ± 0.03 fg	20.65 ± 0.22 h	54.52 ± 0.39 hi	28.62 ± 0.47 j	0.10 ± 0.01 e-h	48.82 ± 0.30 b	< LOQ g	186.68 ± 2.20 g
CONE-CON-21	1.17 ± 0.04 c-e	43.81 ± 1.07 h	48.12 ± 1.23 hi	18.91 ± 0.49 lm	0.81 ± 0.12 b	27.32 ± 0.35 e	< LOQ g	32.13 ± 0.44 k

Data represent the mean ± standard deviation of triplicate extractions (n=3). Letters represent ANOVA post-hoc groupings ($P < 0.05$).

CAL-Calypso. **CAS**-Cascade. **CON**-Contessa. **FL**-Flower stage, **MID**-Middle stage, **HV**-Harvest stage. **21**-2021 crop year. **22**-2022 crop year.

Chlorgenic and phenolic acids

Chlorogenic acids were also generally more prevalent in hop leaves than cones and were present in significantly greater quantities than phenolic acids for all materials evaluated

(Table 6.2, Figure 6.5). Total chlorogenic acids quantified ranged from 11.8-135.7 mg/100 g DM in leaves and 26.8-55.2 mg/100 g DM in cones whilst phenolic acids for those quantified ranged from 0.95-3.35 mg/100 g DM in leaves and 2.63-2.80 mg/100 g DM in cones (Table 6.2). Chlorogenic acids have previously been identified in hop leaves by Morcol et al. (2021) and in South African hop leaves in Chapter 4. However, this is the first time they have been quantified and confirmed to be major phenolic components second only to the flavonol glycosides.

Table 6.2: Chlorogenic and phenolic acids contents in hop leaves (LF) and cones (CONE) (mg/100 g DM).

	Protocatechuic acid	Coumaric acid	Ferulic acid	Chlorogenic acid	Neochlorogenic acid	5-O-Feruloylquinic acid	4,5-Dicaffeoylquinic acid
LF-CAL-Y-21	0.49 ± 0.05 f	1.99 ± 0.03 a	0.87 ± 0.04 a	11.48 ± 0.56 gh	46.34 ± 1.39 c	9.14 ± 0.50 g	1.52 ± 0.04 hi
LF-CAL-Y-22	0.48 ± 0.01 f	1.62 ± 0.09 b	0.80 ± 0.01 ab	13.43 ± 0.51 ef	52.12 ± 1.45 b	11.51 ± 0.04 f	1.75 ± 0.06 f-h
LF-CAL-O-22	0.40 ± 0.03 f-h	0.53 ± 0.05 fg	0.71 ± 0.04 b-e	16.58 ± 0.80 b	35.74 ± 1.15 d	10.67 ± 0.40 f	2.33 ± 0.05 c-e
LF-CAL-FL-21	0.24 ± 0.02 h	0.85 ± 0.02 e	0.66 ± 0.06 c-e	9.48 ± 0.18 ij	27.28 ± 1.45 ef	8.50 ± 0.13 g	1.47 ± 0.03 hi
LF-CAL-HV-21	0.27 ± 0.04 gh	0.47 ± 0.03 g	0.59 ± 0.03 e-g	14.28 ± 0.42 de	17.64 ± 0.68 j	7.33 ± 0.16 h	2.06 ± 0.18 e-g
LF-CAL-FL-22	0.65 ± 0.02 e	0.54 ± 0.03 f	0.66 ± 0.01 c-e	12.11 ± 0.40 fg	30.07 ± 0.74 e	14.90 ± 0.77 d	1.39 ± 0.04 hi
LF-CAL-MID-22	0.37 ± 0.04 f-h	0.30 ± 0.00 i	0.49 ± 0.01 gh	10.59 ± 0.27 hi	23.87 ± 0.51 g-i	8.55 ± 0.24 g	2.04 ± 0.02 e-g
LF-CAL-HV-22	0.31 ± 0.03 gh	0.34 ± 0.05 hi	0.47 ± 0.08 gh	10.53 ± 0.25 hi	21.50 ± 0.35 i	6.53 ± 0.14 hi	2.54 ± 0.12 c
CONE-CAL-21	0.42 ± 0.04 fg	1.61 ± 0.02 b	0.78 ± 0.08 a-c	6.11 ± 0.39 m	13.52 ± 0.90 k	5.61 ± 0.10 i	1.51 ± 0.40 hi
LF-CAS-FL-21	0.31 ± 0.07 gh	0.14 ± 0.02 kl	0.49 ± 0.03 gh	16.36 ± 0.09 bc	51.74 ± 0.70 b	15.22 ± 0.36 d	2.11 ± 0.01 d-f
LF-CAS-HV-21	0.33 ± 0.03 f-h	0.40 ± 0.03 h	0.39 ± 0.03 h	37.72 ± 0.12 a	71.19 ± 2.43 a	25.06 ± 0.43 a	1.71 ± 0.06 gh
LF-CAS-FL-22	2.03 ± 0.10 a	0.09 ± 0.01 l	0.37 ± 0.03 h	6.88 ± 0.08 lm	15.43 ± 0.24 jk	13.17 ± 0.27 e	0.54 ± 0.02 j
LF-CAS-MID-22	1.19 ± 0.07 c	0.10 ± 0.01 l	0.41 ± 0.04 h	7.49 ± 0.36 k-m	15.59 ± 0.57 jk	11.11 ± 0.10 f	1.69 ± 0.02 hi
LF-CAS-HV-22	1.28 ± 0.09 c	0.23 ± 0.02 j	0.51 ± 0.04 f-h	7.23 ± 0.23 lm	25.94 ± 0.59 fg	11.22 ± 0.25 f	1.45 ± 0.05 hi
CONE-CAS-21	0.99 ± 0.05 d	0.93 ± 0.03 d	0.81 ± 0.02 ab	15.59 ± 1.20 b-d	16.45 ± 0.67 jk	8.42 ± 0.39 g	1.71 ± 0.11 gh
LF-CON-FL-21	0.34 ± 0.02 f-h	0.19 ± 0.01 jk	0.75 ± 0.05 a-d	15.06 ± 0.82 cd	37.17 ± 1.17 d	18.30 ± 0.10 c	4.20 ± 0.13 a
LF-CON-MID-21	0.72 ± 0.03 e	0.47 ± 0.04 m	0.86 ± 0.08 a	> LOQ n	7.52 ± 0.10 l	> LOQ j	4.27 ± 0.08 a
LF-CON-FL-22	1.58 ± 0.10 b	0.09 ± 0.02 l	0.42 ± 0.04 h	7.92 ± 0.10 kl	18.06 ± 0.06 j	20.23 ± 0.64 b	1.22 ± 0.03 i
LF-CON-MID-22	0.74 ± 0.02 e	0.19 ± 0.04 jk	0.59 ± 0.03 e-g	8.87 ± 0.13 jk	23.27 ± 0.76 g-i	14.67 ± 0.26 d	3.59 ± 0.06 b
LF-CON-HV-22	0.98 ± 0.07 d	0.21 ± 0.01 j	0.45 ± 0.03 h	8.01 ± 0.21 kl	22.76 ± 0.67 hi	15.37 ± 0.28 d	4.05 ± 0.09 a
CONE-CON-21	0.93 ± 0.05 d	1.07 ± 0.05 c	0.64 ± 0.03 d-f	14.61 ± 0.36 de	25.45 ± 0.57 f-h	12.66 ± 0.15 e	2.46 ± 0.22 cd

Data represent the mean ± standard deviation of triplicate extractions (n=3). Letters represent ANOVA post-hoc groupings ($P < 0.05$).

CAL-Calypso. **CAS**-Cascade. **CON**-Contessa. **FL**-Flower stage, **MID**-Middle stage, **HV**-Harvest stage. **21**-2021 crop year. **22**-2022 crop year.

Neochlorogenic acid (3-CQA) was present at highest concentrations in all materials generally followed by chlorogenic acid (5-CQA), 5-O-feruloylquinic acid and 4,5-dicaffeoylquinic acid. Although chlorogenic acid ratios differ depending on the plant tissue and species, 5-CQA is generally the main constituent followed by 3-CQA and 4-CQA, with dicaffeoylquinic acids typically present at lower concentrations (Clifford, 1999).

In terms of varietal differences, Calypso leaves were generally characterised by higher levels of coumaroylquinic acids, 2,6-dihydroxybenzoic acid and lower levels of feruloylquinic and protocatechuic acid. Specifically, CAL-Y leaves exhibited particularly high hydroxycinnamic acids, quinate and ascorbate (Figure 6.5). Contessa leaves were generally distinguished by a higher ratio of dicaffeoylquinic to caffeoylquinic acids. Cascade leaves generally had lower coumaroylquinic, caffeoylquinic and dicaffeoylquinic acids, however clustered differently according to crop year due to significantly higher caffeoylquinic and feruloylquinic acid abundance in 2021 compared to 2022. This trend was not observed in other varieties. Cones were distinguished by higher 4-hydroxybenzoic acid levels and generally lower chlorogenic acid levels (Figure 6.5).

Flavanols and procyanidins

Procyanidins and their respective flavanol monomers have been reported as major components of hop cone polyphenols depending on variety (Li and Deinzer, 2006) and geographical origin (Olšovská et al., 2013). Whilst B-type dimers have been identified in hop leaves (Morcol et al., 2021), procyanidins are quantified for the first time in this study. All cone extracts contained significantly higher amounts of all flavanols (except gallo catechin) and procyanidins compared to all leaf extracts (Table 6.3, Figure 6.5), which is consistent with the results for total PAC shown in Figure 6.3. Total flavanols and procyanidins ranged between 136.2-214.0 mg/100 g DM and 0.64-55.7 mg/100 g DM for cones and leaves respectively (Table 6.3). These amounts are significantly lower than the acid-butanol test proanthocyanidin contents reported in Figure 6.3, which suggests that like hop cones, hop

leaves are predominantly made up of higher molecular weight proanthocyanidins. A mean degree of polymerisation of 7.8 was found for Willamette cones (Taylor et al., 2003) whilst residue from supercritical CO_2 extraction of Saaz T90 pellets contained proanthocyanidins at molecular weights up to 100 kDa (Gadon et al., 2019a).

Table 6.3: Flavanols and procyanidins in hop leaves (LF) and cones (CONE) (mg/100 g DM).

	Catechin	Epicatechin	Procyanidin B1	Procyanidin B2	Procyanidin B3	Procyanidin C1
LF-CAL-Y-21	10.66 ± 0.48 ef	0.49 ± 0.03 ef	1.00 ± 0.05 e-g	0.19 ± 0.02 e	2.05 ± 0.23 de	< LOQ e
LF-CAL-Y-22	42.60 ± 0.89 c	2.20 ± 0.06 c	3.55 ± 0.07 d	0.48 ± 0.07 e	6.59 ± 0.06 c	< LOQ e
LF-CAL-O-22	10.17 ± 0.38 f	0.72 ± 0.08 e	3.26 ± 0.15 d	0.46 ± 0.06 e	2.96 ± 0.05 de	< LOQ e
LF-CAL-FL-21	7.06 ± 0.23 g	0.32 ± 0.02 ef	0.31 ± 0.06 g	0.21 ± 0.03	< LOQ e	< LOQ e
LF-CAL-HV-21	3.10 ± 0.11 i-k	0.24 ± 0.03 ef	0.71 ± 0.10 fg	0.25 ± 0.01 e	< LOQ e	< LOQ e
LF-CAL-FL-22	3.46 ± 0.34 h-j	0.20 ± 0.04 ef	0.95 ± 0.06 e-g	0.21 ± 0.04	1.10 ± 0.16 de	< LOQ e
LF-CAL-MID-22	5.88 ± 0.22 gh	0.35 ± 0.00 ef	2.29 ± 0.06 d-f	0.32 ± 0.07	1.84 ± 0.11 de	< LOQ e
LF-CAL-HV-22	4.39 ± 0.20 hi	0.20 ± 0.01 ef	2.47 ± 0.08 de	0.30 ± 0.03	1.37 ± 0.12 de	< LOQ e
CONE-CAL-21	77.40 ± 3.23 b	7.73 ± 0.34 b	23.74 ± 1.29 b	2.31 ± 0.15 c	39.58 ± 4.26 b	3.08 ± 0.19 c
LF-CAS-FL-21	4.36 ± 0.19 hi	1.54 ± 0.12 d	1.43 ± 0.07 efg	0.44 ± 0.02 e	1.33 ± 0.04 de	< LOQ e
LF-CAS-HV-21	4.34 ± 0.16 hi	0.70 ± 0.07 e	0.63 ± 0.13 fg	0.16 ± 0.01	0.37 ± 0.06 de	< LOQ e
LF-CAS-FL-22	1.24 ± 0.08 jk	0.16 ± 0.01 f	0.60 ± 0.02 fg	0.19 ± 0.02	0.37 ± 0.07 de	< LOQ e
LF-CAS-MID-22	1.02 ± 0.07 jk	0.15 ± 0.05 f	1.04 ± 0.11 e-g	0.25 ± 0.07	0.39 ± 0.11 de	< LOQ e
LF-CAS-HV-22	1.29 ± 0.04 jk	0.23 ± 0.02 ef	0.84 ± 0.02 e-g	0.23 ± 0.02 e	0.25 ± 0.04 e	< LOQ e
CONE-CAS-21	41.69 ± 1.27 c	19.52 ± 0.59 a	22.03 ± 1.29 b	7.00 ± 0.36 a	38.61 ± 1.87 b	7.13 ± 0.02 a
LF-CON-FL-21	15.84 ± 0.07 d	1.77 ± 0.08 cd	5.85 ± 0.27 c	1.08 ± 0.14 d	7.36 ± 0.35 c	0.54 ± 0.11 de
LF-CON-MID-21	0.64 ± 0.04 k	< LOQ f	< LOQ g	< LOQ e	< LOQ e	< LOQ e
LF-CON-FL-22	5.95 ± 0.35 gh	0.38 ± 0.03 ef	3.64 0.10 ± d	0.63 ± 0.06 e	3.91 ± 0.54 cd	0.50 ± 0.14 d
LF-CON-MID-22	12.98 ± 0.20 e	0.71 ± 0.02 e	7.28 ± 0.42 c	1.06 ± 0.00 d	6.93 ± 0.33 c	0.71 ± 0.14 d
LF-CON-HV-22	8.30 ± 0.18 fg	0.44 ± 0.07 ef	3.91 ± 0.10 d	0.52 ± 0.06 e	2.30 ± 0.13 de	< LOQ e
CONE-CON-21	103.68 ± 1.09 a	7.55 ± 0.29 b	37.89 ± 1.69 a	3.94 ± 0.18 b	56.09 ± 2.41 a	4.87 ± 0.08 b

Data represent the mean ± standard deviation of triplicate extractions (n=3). Letters represent ANOVA post-hoc groupings ($P < 0.05$).

CAL-Calypso. **CAS**-Cascade. **CON**-Contessa. **FL**-Flower stage, **MID**-Middle stage, **HV**-Harvest stage. **21**-2021 crop year. **22**-2022 crop year.

The main flavanols and proanthocyanidins for all cone and leaf extracts were catechin, epicatechin and B-type procyanidins B1 and B3 with B2 and C1 present at lower concentrations whilst A1/A2 were < LOD for all materials (Table 6.3). Although A-type PACs have been detected in the leaves of some species such as Rubiaceae coffee (Cangeloni et al.,

2022), generally B-type PACs are more abundant. Catechin was the most abundant compound in this class for both leaves and cones. Cascade cones and leaves across stage of development and crop year had lower catechin: epicatechin ratios (2.1-7.9) compared to Contessa (>8.9) and Calypso (10.1-22.1). Total flavanols, in parallel with findings for chlorogenic acids, were significantly lower for Cascade in 2022 compared to 2021; however, this trend was not observed for other varieties.

Prenylflavanoids and α- / β-acids

As expected prenylflavonoid and bitter acid levels were significantly higher in hop cones (3.37-10.6 g total/100g DM) than in leaves (< 300 mg total/100g DM) (Table 6.4, Figure 6.5) as both are predominantly synthesised in the lupulin glands of hop cones which contain enzymes responsible for prenylation (Dostálek et al., 2017). Bitter resins and prenylflavonoids are the most abundant secondary metabolites in hop cones (up to 300 mg/g DM) (Almaguer et al., 2014). As for hop cones, xanthohumol was the principal prenylflavonoid in hop leaves with iso-xanthoumol and 6-PN found in lower concentrations. In contrast to hop cones, leaf β-acids were present in greater amounts than α-acids in all samples other than CAL-HV-21 (Table 6.4). Four types of glandular trichomes have been identified on the abaxial side of hop leaves and tendrils which differ morphologically from cone trichomes (Gülz et al., 1993). Hop leaf trichome density has been positively correlated to abundance of prenylflavonoids, bitter resins and co-multifidol glucoside, the aglycone of which is a precursor of bitter acid biosynthesis (Morcol et al., 2021). It is thus suggested that the prenylflavonoids and bitter resins identified in this study may be present in leaf glandular trichomes whilst the more ubiquitous sub-groups such as phenolic acids, flavonol glycosides and proanthocyanidins are present predominantly in non-glandular epidermal layers of green leaf tissue (Winkel-Shirley, 2002).

Table 6.4: Prenylflavonoids and bitter resin contents of hop leaves (LF) and cones (CONE) (mg/100 g DM).

	Isoxanthohumol	6-PN	Xanthohumol	Cohumulone	Ad/Humulone	Colupulone	Ad/Lupulone
LF-CAL-Y-21	0.20 ± 0.01 f	0.67 ± 0.03 a	9.60 ± 0.30 e-g	< LOQ c	< LOQ c	9.21 ± 3.26 ef	31.66 ± 7.48 ef
LF-CAL-Y-22	0.55 ± 0.00 b	0.61 ± 0.02 b	18.32 ± 0.26 a	< LOQ c	< LOQ c	20.12 ± 1.02 c	58.41 ± 2.73 c
LF-CAL-O-22	0.57 ± 0.04 ab	0.34 ± 0.02 e	12.89 ± 0.55 c	< LOQ c	< LOQ c	19.19 ± 0.92 cd	44.94 ± 0.21 d
LF-CAL-FL-21	0.25 ± 0.02 ef	0.30 ± 0.01 ef	6.16 ± 0.11 i	< LOQ c	< LOQ c	4.42 ± 1.48 fg	15.91 ± 3.90 g
LF-CAL-HV-21	0.30 ± 0.01 de	0.41 ± 0.02 d	6.70 ± 0.16 hi	11.91 ± 2.21 b	27.48 ± 4.19 b	13.44 ± 2.06 de	23.80 ± 2.94 fg
LF-CAL-FL-22	0.25 ± 0.01 ef	0.26 ± 0.02 fg	11.80 ± 0.18 d	< LOQ c	< LOQ c	15.32 ± 1.33 c-e	34.86 ± 2.84 e
LF-CAL-MID-22	0.56 ± 0.05 ab	0.29 ± 0.01 ef	10.16 ± 0.13 e	< LOQ c	< LOQ c	11.62 ± 1.14 e	28.04 ± 2.34 ef
LF-CAL-HV-22	0.47 ± 0.03 c	0.30 ± 0.01 ef	9.00 ± 0.16 fg	< LOQ c	< LOQ c	11.62 ± 0.14 e	28.17 ± 0.89 ef
CONE-CAL-21	9.50 ± 0.38	7.47 ± 0.39	505.57 ± 2.12	4079.99 ± 39.62	5130.28 ± 59.19	523.38 ± 14.49	297.77 ± 0.10
LF-CAS-FL-21	0.24 ± 0.01 ef	0.18 ± 0.01 h	7.40 ± 0.22 h	< LOQ c	< LOQ c	19.69 ± 1.00 cd	32.85 ± 0.68 ef
LF-CAS-HV-21	0.41 ± 0.02 c	0.52 ± 0.02 c	15.03 ± 0.66 b	37.17 ± 0.93 a	52.57 ± 4.89 a	78.78 ± 4.44 a	83.92 ± 2.47 a
LF-CAS-FL-22	0.33 ± 0.03 d	0.22 ± 0.01 gh	7.16 ± 0.42 hi	< LOQ c	< LOQ c	44.89 ± 2.95 b	60.49 ± 3.35 bc
LF-CAS-MID-22	0.46 ± 0.01 c	0.29 ± 0.02 f	8.65 ± 0.26 g	< LOQ c	< LOQ c	47.07 ± 0.95 b	65.94 ± 0.47 bc
LF-CAS-HV-22	0.62 ± 0.02 a	0.29 ± 0.01 f	9.89 ± 0.09 ef	< LOQ c	< LOQ c	49.77 ± 5.38 b	69.68 ± 6.75 b
CONE-CAS-21	3.90 ± 0.12	6.05 ± 0.09	244.56 ± 9.19	1191.18 ± 82.51	2869.08 ± 179.35	720.75 ± 42.39	761.21 ± 26.60
LF-CON-FL-21	< LOQ g	< LOQ j	0.49 ± 0.04 k	< LOQ c	< LOQ c	< LOQ g	< LOQ h
LF-CON-MID-21	< LOQ g	0.06 ± 0.03 i	1.30 ± 0.16 jk	< LOQ c	< LOQ c	< LOQ g	< LOQ h
LF-CON-FL-22	< LOQ g	< LOQ j	1.03 ± 0.09 jk	< LOQ c	< LOQ c	< LOQ g	< LOQ h
LF-CON-MID-22	0.01 ± 0.01 g	0.02 ± 0.01 ij	1.40 ± 0.04 jk	< LOQ c	< LOQ c	< LOQ g	< LOQ h
LF-CON-HV-22	0.02 ± 0.01 g	0.03 ± 0.01 ij	1.71 ± 0.06 j	< LOQ c	< LOQ c	1.51 ± 0.00 g	2.40 ± 0.00 h
CONE-CON-21	5.79 ± 0.13	3.09 ± 0.06	195.36 ± 7.84	457.46 ± 17.62	969.01 ± 36.54	841.86 ± 35.51	896.05 ± 34.38

Data represent the mean ± standard deviation of triplicate extractions (n=3). Letters represent ANOVA post-hoc groupings ($P < 0.05$) (cone data omitted).

CAL-Calypso. **CAS**-Cascade. **CON**-Contessa. **FL**-Flower stage, **MID**-Middle stage, **HV**-Harvest stage. **21**-2021 crop year. **22**-2022 crop year.

Leaf total resin and prenylflavonoid levels for both crop years followed the same order of abundance: Cascade > Calypso > Contessa, although proportions of these compounds differed. Contessa leaves were particularly low in resins and prenylflavanoids whilst Cascade and Calypso were more similar but could generally be distinguished by their bitter acid: prenylflavonoid ratios which were higher for Cascade (6.7-15.6) compared to Calypso (3.0-10.3). For Contessa and Cascade leaves, prenylflavonoids and bitter resins accumulated progressively over the course of development for both crop years, whereas the trends

observed for Calypso were more variable. α-acids were only present at concentrations above the LOQ in two leaf samples at harvest. α-acids may thus be synthesised later than β-acids in leaves whereas accumulation patterns of α- and β-acids for cones have been shown to be similar (Kavalier et al., 2011).

7.4.4-Multivariate analysis of hop leaf phenolic data

Variety, developmental stage, crop year, leaf age and climate conditions such as heat and UV exposure are factors which impact upon leaf phenolic content and therefore were a focus of this study (Winkel-Shirley, 2002). To investigate impacts of these factors on hop leaf phenolic profile, data were subjected to principal component analysis (PCA), hierarchical cluster analysis (HCA) and correlation analysis. Cone data and phenolic variables with IS normalised abundance < 0.1 in the highest sample were omitted from these analyses. PCA generated seven principal components with eigenvalues > 1 explaining 94.8% of variation which were analysed using Wards hierarchical clustering method (Euclidean distance) to measure dissimilarity based on phenolic variables. The dendrogram showing clustering of samples into groups is presented in Figure 6.6.

Two clusters were formed based on Hartigan index truncation which separated samples by variety with Calypso samples in C1 (blue) and Contessa and Cascade samples in C2 (red). C1 was more homogenous than C2 which sub-clustered according to variety with Contessa and Cascade grouped separately. CAL-Y samples in C1 were most dissimilar (especially CAL-Y-22), whilst 2021 samples were most dissimilar in the C2 Cascade subcluster with 2022 Cascade samples much less variable. Contessa samples subclustered seemingly independently of the factors evaluated in this study.

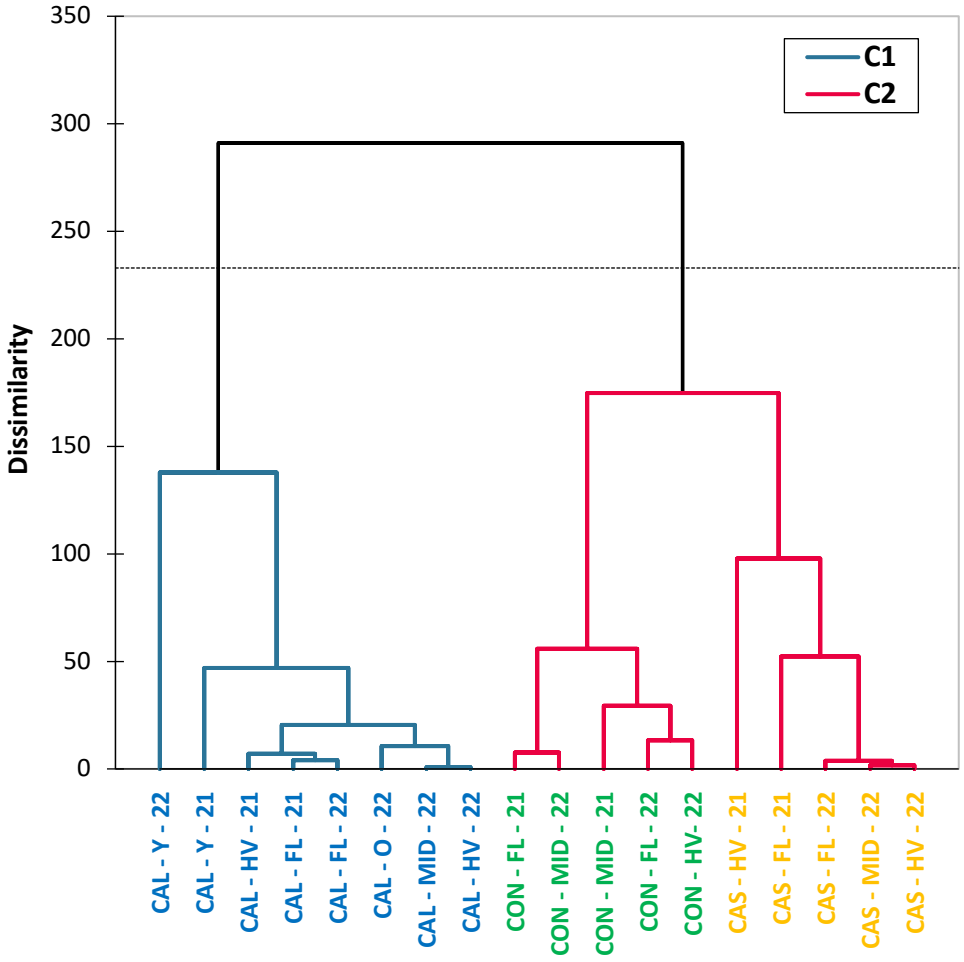


Figure 6.6: Dendrogram of cluster analysis of hop leaves based on phenolic profile. Samples coloured according to variety; Calypso-blue, Contessa-green, Cascade-orange.

The PCA biplot for PC1 and PC2 (Figure 6.7) explains 58.3% of the variability in the data set (PC1-36.1%, PC2-22.2%). The observations plot shows groupings based on leaf variety with some crop year, but not stage of development, patterns. Generally, leaf samples from 2022 group together more closely than for 2021 however only within and not between varieties. This confirms, as was apparent from the heat map shown in Figure 6.5, that variety was the most significant factor determining hop leaf polyphenol composition. Some leaf age and variety specific crop year effects were also observed but there was no obvious clustering according to stage of development.

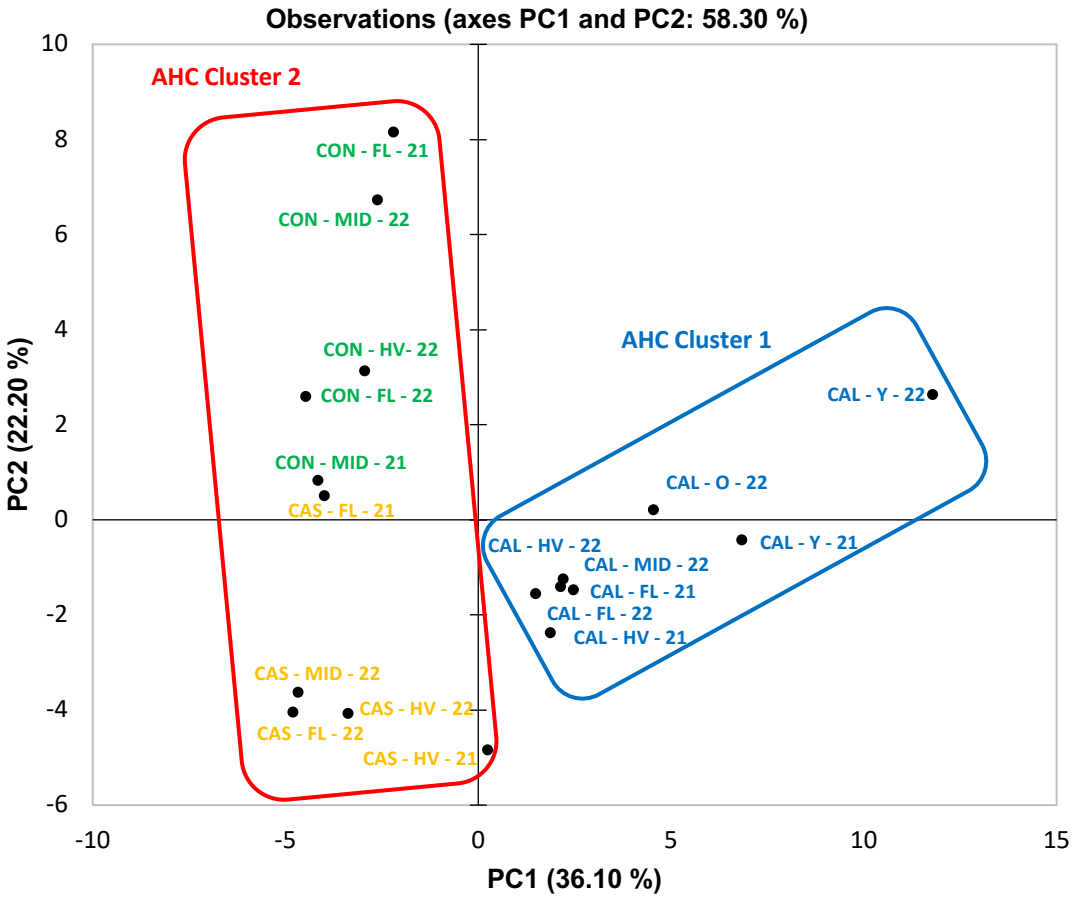


Figure 6.7: PCA observations plot of hop leaves based on phenolic profile. Coloured according to variety for Calypso-blue, Cascade-orange and Contessa-green. Clustering according to dendrogram in Figure 6.6.

To investigate which phenolics are driving sample groupings, correlations between phenolic variables and principal components were investigated. PC1 was strongly positively correlated to quercetin glycosides as well as K-GALAC, K-NEO, DMX and COQA A. PC1 was negatively correlated to K-3-R-7,4-DIGALAC, K-RUT, K-3,7-DILGLUC and clitorin. PC2 was strongly positively correlated with concentrations of K-MG, K-GLUC and procyanidins B1, B2 and B3 whilst negatively correlated to bitter acid and prenylflavonoid concentrations.

Overall, flavonol glycosides were the main compounds driving varietal differences with Calypso distinguished along PC1 by higher levels of quercetin glycosides and lower levels of kaempferol glycosides. Young leaf samples loaded most positively on PC1, due in particular to their elevated concentrations of flavonol glycosides. Contessa was distinguished from

Cascade along PC2 by its higher levels of malonylated and mono kaempferol glycosides and procyanidins but lower levels of di- and tri-kaempferol glycosides, prenylflavonoids and bitter acids. Glycoside profiles have been used for cultivar discrimination in cones (Dušek et al., 2021) and to distinguish ssp. *lupulus* accessions from native ssp. *lupuloides* accessions in hop leaves (McCallum et al., 2019). Analysis presented in this study indicates leaf glycoside profiles could be used for cultivar discrimination whereas caffeoylquinic acids, although quantitatively important, were less variety specific.

PC3 (data not shown) mainly separated the differences between the two harvest years for Cascade leaves and was most positively correlated with abundance of clitorin, 3-CQA and 5-CQA which were present in greater amounts in 2021.

6.4.5-Impacts of climatic variables

To investigate potential impacts of climate conditions on phenolic compound accumulation, 7-day averages for average temperature and solar radiation prior to leaf collection were analysed using Pearson's correlation analysis separately for each variety across the developmental stages FL, MID and HV. A correlation matrix is provided in Appendix 6, Table 1. Heat and UV exposure are thought to be some of the main plant stress factors that impact plant flavonoid synthesis (Winkel-Shirley, 2002). For solar radiation significant positive correlations were found with concentrations of ascorbate, quinate, coumaroylquinic acid (A) and Q-MG for Calypso and K-3-R-7,4-DIGALAC for Contessa. For temperature, significant positive correlations were found for proanthocyanidin content for Cascade whilst significant negative correlations were found for hulupinic acid, colupulone, feruloylquinic acid (G), phloroglucinol glycoside-C for Cascade and protocatechuic acid for Contessa. Correlations were not consistent within phenolic sub-groups or between varieties however bitter acids and prenylflavonoids were generally negatively correlated to solar radiation and temperature for all varieties.

Drought stress has also been shown to have an effect on hop leaf and cone polyphenols (Ceh et al., 2007), however as this farm was fully irrigated and total precipitation was consistently low for both crop years this factor was not investigated.

6.4.6-Potential for valorisation of hop leaf phenolics

This study presents the first comprehensive quantitative analysis of phenolics in hop leaves sampled across two harvest years for three different varieties and at different stages of leaf development. Flavonol glycosides and chlorogenic acids are identified as the dominant components. Phenolic concentrations are expressed on a dry matter basis and represent the total extracted using a simple, low-cost extraction protocol that could readily be scaled up. Extractions used a 'green', widely available solvent, with maceration and high solid: liquid ratios for increased capacity and lower solvent and treatment costs.

Quantitatively, quercetin and kaempferol glycosides were by far the most abundant groups. In particular K-RUT, Q-RUT, Q-MG and Q-GLUC concentrations exceeded or were comparable to those reported in other notable plant sources of flavonol glycosides such as mulberry (Ju et al., 2018) and Ginkgo leaves (Gray et al., 2006). Similarly, total chlorogenic acid levels in hop leaves were found to be comparable to those reported in spent coffee grounds (Al-Dhabi et al., 2017), and other agri-food materials that have been evaluated as a source of chlorogenic acids (Frosi et al., 2021). Attempts to valorise hop leaves as sources of phenolics must consider the bioactive properties of these compounds and the most effective purification technique. Numerous studies have highlighted the antioxidant properties of chlorogenic acids (Tošović et al., 2017) and flavonol glycosides (Leong et al., 2008), indicating a potential use of hop leaf extracts in improving the shelf life and nutritional properties of foods and beverages. Quercetin 3-O-glycosides from mulberry leaves have demonstrated strong LDL antioxidant activity, potentially reducing atherosclerosis (Yang et al., 2018). O-glycosylation can enhance bioavailability however it has been shown to decrease flavanol

antioxidant activity depending on the number, position, and type of sugar moiety as well as the aglycone (Heim et al., 2002). This highlights the importance of investigating whether hydrolysis of hop leaf extracts would increase their antioxidant activity and therefore commercial value. In extracts of other plant tissues enzymatic hydrolysis has been shown to be more effective than acid hydrolysis for removal of the glycoside moiety to improve antioxidant activity (Wang et al., 2011).

Variation in phenolic composition of leaf samples is also an important consideration for industrial production of extracts. This study indicates that cultivar is the most significant factor determining leaf phenolic composition, with crop year and climate being less significant. Therefore, it seems cultivar is the most important factor to control for leaf harvesting for consistent phenolic content and therefore bioactive properties. Comparisons of the leaf extracts reported in this chapter with South African leaf extracts of different varieties analysed in Chapter 6 further highlights the phenolic variability of hop leaves, although it is unclear if this is attributable to variety.

Another important consideration is the efficacy, cost and carbon footprint of different drying techniques. Hop leaves post-harvest have a high moisture content and require stabilisation to prevent degradation and maintain bioactive properties. This could be performed using the cone kilning facilities located at the hop farm, however kilning is the single most significant contributor to the global warming potential of hop production (28.19%) (Bristol, 2022). Recent research also demonstrated that kiln dried hop leaves exhibited lower antioxidant activity compared to the respective freeze-dried leaves (Macchioni et al., 2021). All such aspects must be considered and optimised before valorisation of hop leaf phenolics becomes commercial reality. However, the present study indicates hop leaves are a promising agri-food residue rich in flavonol 3-O-glycosides and chlorogenic acids which could be used for the development of new value-added products for the food and beverage industries.

Chapter 7-Hop leaf phenolics as a source of natural antioxidants.

7.1-Introduction

The results presented in Chapters 4 and 6 highlighted that hop leaves are a particularly rich source of quercetin and kaempferol 3-O-glycosides with composition mainly dependant on variety. However, flavonol 3-O-glycosides exhibit weaker antioxidant properties compared to their respective aglycones depending on the flavonol as well as the glycoside type, number, and position on the phenolic structure (Xiao et al., 2021). This reduced antioxidant potential may limit the value of hop leaf extracts as effective natural antioxidants in various applications. For example, Chapter 5 highlighted that high value commercial phenolic extracts; Grape OPC, Pineol and Vitisol exhibited higher antioxidant activity compared to that of the pure compounds quercetin/kaempferol 3-O-glycoside. This necessitates the need to evaluate hydrolytic treatment of flavonol 3-O-glycoside rich leaf extracts for improved antioxidant properties to support the development of purified extracts with comparable antioxidant activity to that of high value commercial extracts.

Different treatments have been evaluated to improve the bioactive properties of flavonols through glycoside hydrolysis, including microbial fermentation (König et al., 2023), acid hydrolysis, and subcritical water hydrolysis (Ravber et al., 2015). However, these are generally too expensive, intensive (in terms of temperature and pH) or impractical for industrial use. Recently enzymatic hydrolysis has emerged as the most effective and 'green' technique (Wang et al., 2011). Specific enzymes have been shown to be effective for hydrolysis of particular glycosides, for example, hesperidinase for rutin hydrolysis (de Araújo et al., 2013). However, hop leaf extracts contain a structurally diverse range of flavonol glycosides which means that a preparation containing a mixture of hydrolysing enzymes may be more suited.

Snailase, an enzyme cocktail derived from the internal organs of Limax (air-breathing land slugs) contains cellulases, pectinases and proteases. It has emerged as one of the most promising enzyme preparations for the hydrolysis of flavonol glycosides in plant extracts (Kornpointner et al., 2022), exhibiting high hydrolytic activity across a range of structurally diverse phenolic glycosides, even at lower temperatures and with shorter incubation times. Table 7.1 summarises the current research on snailase-assisted glycoside hydrolysis, highlighting the reported optimal incubation conditions and its effect on the resulting bioactivity.

Table 7.1: Summary of previous studies on optimal incubation conditions for snailase hydrolysis of glycosides and its impact on aglycone liberation and bioactivity.

Extract / Substrate	Snailase conditions evaluated	Conclusions	Reference
Icariin	37 °C 1-12 h pH 6.8	-Icariin almost completely hydrolysed within 4 h snailase incubation resulting in significantly improved oral bioavailability.	**(Liu et al., 2017).**
Leaf and flower extracts	37 °C 25 min pH 5.5	-Snailase more effective than comparable enzymes and acid hydrolysis for crude leaf and flower extracts for a variety of flavonoid subgroups.	**(Kornpointner et al., 2022).**
Apple pomace extract	0-20%, 0-3 g/15 ml snailase 1 h pH 5 37 °C -Shaken (180 rpm)	-Snailase can remove sugar moieties from kaempferol and quercetin glycosides, phloridzin and 3-hydroxyphloridzin. -These can be extracted with supercritical CO_2 and small amounts of polar cosolvents.	**(Mikšovsky et al., 2023).**
Rutin	40 °C 12-48 h	-Snailase was more effective than acid treatment for rutin hydrolysis and production of quercetin and quercetin 3-O-glucoside. -Meta ions all had depressant effects on conversion of rutin to quercetin.	**(Wang et al., 2011, Wang et al., 2012).**
Epimedin C from *E. wushanense* extract	35-70 °C 0-12 h pH 4-7 0-10 mg snailase/ml	-Snailase was more effective than comparable enzymes for production of rhamnosyl Icariside II. -Optimal conditions were 4 h at 55 °C in sodium acetate buffer at pH 5.5. -Extract purification not necessary for snailase treatment.	**(Chen et al., 2024).**
Polydatin from *P. cuspidatum* extract	40-70 °C 30-180 min pH 4.5 (sodium acetate buffer) 0-10 mg snailase/ml 2-10% snailase load	-Optimised conditions fully convert polydatin to resveratrol for medicinal purposes. -Optimised conditions were determined to be 62 °C, 96 min reaction time and 6.6% enzyme load by response surface methodology.	**(Wang et al., 2013).**
Phenolic acid glycosides from *G. biloba* extract	37 °C 36 h	-Snailase is also effective for hydrolysis of phenolic acid glycosides. Aglycones included protocatechuic, p-hydroxybenzoic, vanillic and p-coumaric acid.	**(Li et al., 2020).**
Icariin	pH 2-8 50–100 °C	-Snailase hydrolysis of epimedium flavonoids resulted in enhanced antitumour activity.	**(Liu et al., 2019).**
Esculentoside B from *R. phytolaccae* extract	37 °C 2 h pH 7.5	-Optimal conditions; 48.28 °C, pH 6.4, enzyme load 4.43%, and reaction time 2.73 h.	**(Cui et al., 2016).**
Flavonol glycosides from *M. nigra* extract	1: 8-1: 12 material: liquid ratio 1-3 extractions 60-120 min	-Optimal conditions; 86.3 min, material-liquid ratio of 1: 10.1, number of extractions of 2.32. -Snailase hydrolysis of flavonol glycosides from Mulberry leaves could enhance the anti-inflammatory activity for zebrafish inflammatory bowel disease.	**(Jia et al., 2024).**

In Chapter 6 hop leaf extracts were characterised for their phenolic content across three varieties, three stages of development and over two crop years. In the present chapter the objectives were: i) to characterise these hop leaf extracts and phenolic standards for their antioxidant activities and ii) to evaluate snailase-assisted hydrolytic treatment of purified hop leaf extracts and phenolic standards for improved antioxidant activity.

7.2-Materials

7.2.1-Chemicals

Snailase was obtained from Abbexa (Cambridge, UK). Sep-Pak C18 (1 g) cartridges were obtained from Waters. Acetic acid and anhydrous sodium acetate (reagent grade) were obtained from Sigma Aldrich. All other chemicals and reference standards were sourced according to 2.1.1.

7.2.2-Hop Materials and Extracts

Hop leaf and cone extracts analysed in this study for antioxidant activity were the same extracts previously characterised for their phenolic content in Chapter 6. This included leaves for three varieties (Calypso-CAL, Cascade-CAS and Contessa-CON), across three stages of development (Flower-FL, Middle-MID and Harvest-HV) for two crop years (2021 and 2022). Young (Y) and old (O) leaves for Calypso were collected based on size for both crop years, and respective cones (CONE) were collected for all varieties for crop year 2021.

For snailase hydrolysis trials, leaf material from S. Aroma, S. Passion (2021 crop) and Cascade (2021 crop year, flower stage of development) was sourced as outlined in Chapter 2.1.2 and 6.2.2 respectively.

7.3-Methods

7.3.1- Hop leaf phenolic extraction and purification for snailase treatment

Three hop leaf samples (CAS-FL-21, S. Aroma-HV-21 and S. Passion-HV-21) were selected for extraction and solid-phase extraction (SPE) purification for subsequent snailase hydrolysis. In brief, 25 g milled hop material was extracted with 250 ml E50 and filtered using vacuum filtration as described in section 2.1. Ethanol was removed using a Heidolph HeiVap rotary evaporator (Schwabach, Germany) at 25 °C for 1 h, and the resulting solution was centrifuged for 10 min at 4000 rpm and the non-dissolvable material was discarded.

Hop extracts were purified using Sep-Pak C18 (1 g) Vac 6 cc solid-phase extraction cartridges with a Waters SPE manifold. Cartridges were equilibrated with ethanol (5 ml) and conditioned with deionised water (5 ml). Hop extract was then loaded (20 ml) onto the cartridge which was subsequently washed with deionised water (5 ml) and dried for 2 min. Ethanol (5 ml) was then used for desorption at a rate of 1 drop/s and the resulting solution underwent rotary evaporation under vacuum at 25 °C for 30 min until 'dry'. Dry material yield was calculated based on flask weight difference, and the purified leaf extracts were stored at -80 °C prior to enzyme treatment.

7.3.2-Snailase hydrolysis of hop leaf phenolic extracts and pure compounds

7.3.2.1 Sodium acetate buffer solution

0.025 M sodium acetate buffer solution (pH 5.5) was produced by mixing 482.6 mg sodium acetate and 22.03 mg acetic acid with 250 ml UP water. pH was adjusted to 5.5 using 0.1 M HCl and NaOH solutions.

7.3.2.2-Snailase Incubation

The purified leaf extracts and standards prepared for snailase treatment are outlined in Table 7.2.

Table 7.2: Purified leaf extracts and phenolic standards for snailase treatment.

Number	Materials	Purification	Purity	g/mol
	Standard Compounds			
1	Quercetin 3-O-rutinoside (Q-RUT)	-	≥ 99	610.52
2	Quercetin 3-O-sophoroside (Q-SOP)		≥ 98	626.50
3	Quercetin 3-O-(6''-malonylglucoside) (Q-MG)		≥ 85	550.42
4	Quercetin 3-O-glucoside (Q-GLUC)		≥ 98	464.10
5	Kaempferol 3-O-rutinoside (K-RUT)		≥ 98	594.52
6	Kaempferol 3-O-glucoside (K-GLUC)		≥ 98.5	448.38
7	Kaempferol 3-O-galactoside (K-GALAC)		≥ 98	448.38
	Hop Leaf Extracts			
8	Cascade (FL-21) Leaves	C18 SPE	-	-
9	S. Passion (HV-20) Leaves	C18 SPE		
10	S. Aroma (HV-20) Leaves	C18 SPE		

For preparation, standards (0.2 mg) were solubilised in 50 µl of ethanol and diluted with 1150 µl 0.025 M acetate buffer to a concentration of 0.17 mg/ml and ethanol content of 4.17% (v/v). For the standards K-RUT and Q-MG only a portion of less than 0.2 mg was available. These were solubilised and diluted with the same volume of ethanol and buffer solution. The C18 purified leaf extracts were solubilised in E50 to 125 mg/ml and diluted with 0.025 M acetate buffer to a concentration of 0.59 mg/ml and an ethanol content of 4.17% v/v.

Snailase was solubilised in 0.025 M acetate buffer for a working solution of 50 mg/ml. For enzymatic treatment 40 µl snailase solution was added to 360 µl sample solution which was then vortexed and incubated in a water bath at 37 °C for 90 min. After enzyme incubation, 400 µl ethanol was added to each sample (final concentration of 0.27 mg/ml) and the resulting solutions were vortexed (10 s) and sonicated for 10 min for enzyme denaturation and to solubilise the aglycone products of hydrolysis. Samples were then centrifuged at 12,000 rpm at room temperature for 10 min to remove the precipitate (as performed by Li et al. (2020)), and stored at -80 °C prior to analysis of antioxidant activity and phenolic content.

7.3.3-Antioxidant Analysis of Extracts

The antioxidant activity of the extracts was evaluated using the DPPH, FRAP, and ORAC assays, as described in Section 2.2.5. Antioxidant activity was expressed as Trolox equivalents for each assay (mg TE/g).

For Ferric Reducing Antioxidant Power (FRAP) analysis crude hop leaf and cone extracts were analysed at a concentration of 2 mg/ml E50. Snailase-treated and control purified leaf extracts were analysed at 0.281 mg/ml E50, whilst snailase-treated and control phenolic standards were analysed at 0.075 mg/ml E50.

For DPPH radical scavenging activity % (RSA) analysis crude hop leaf and cone extracts were analysed at six different concentrations between 0.03-1.5 mg/ml E50 which were used to calculate IC50 values. The RSA of leaf and cone extracts at six concentrations are provided in

Appendix 7, Figures 1 and 2 respectively. Snailase-treated and control extracts were analysed in duplicate at 0.0375 mg/ml, whilst phenolic standards were analysed in duplicate at 0.01 mg/ml.

To analyse the oxygen radical absorbance capacity (ORAC) analysis, crude hop leaf and cone extracts were analysed at 0.1 mg/ml phosphate buffer solution (PBS) pH 7.4, whilst purified leaf extracts were analysed at 0.008 mg/ml 50% aqueous acetone (v/v) (A50). Snailase-treated and control extracts, along with phenolic standards, were analysed at 0.005 mg/ml A50. Standards of flavonols and their glycosides were additionally analysed at concentrations of 0.00375 mg/ml and 0.0025 mg/ml A50, with results averaged across all three concentrations. The Trolox equivalents of for all three concentrations expressed on a weight basis are provided in Appendix 7, Figure 3.

7.3.4- LC-ESI-qTOF-MS/MS

Phenolic analysis of snailase treated and control leaf extracts was performed using LC-ESI-qTOF-MS/MS as outlined in 2.2.6.2. Extracts were syringe filtered at 0.22 µm, diluted with E50 and analysed at 50 µg/ml. IS solution comprised of 18 candidate phenolic compounds for a final concentration of 2.5 µg of each candidate/ml E50 as detailed in Chapter 6. Hop extracts were analysed in duplicate per treatment (four replicates per condition) and a QC sample comprising equal parts of each extract was analysed after every 12 sample injections to check system performance. Quantitative analysis was performed using standard curves outlined in Appendix 6-Table 6, whilst relative quantitation was performed by normalising peak areas against that of hesperidin.

7.4-Results and Discussion

7.4.1-Antioxidant Activities of hop leaf and cone samples

The FRAP, DPPH and ORAC antioxidant activities of hop leaf and cone extracts analysed for their phenolic contents in Chapter 6, are presented in Figure 7.1.

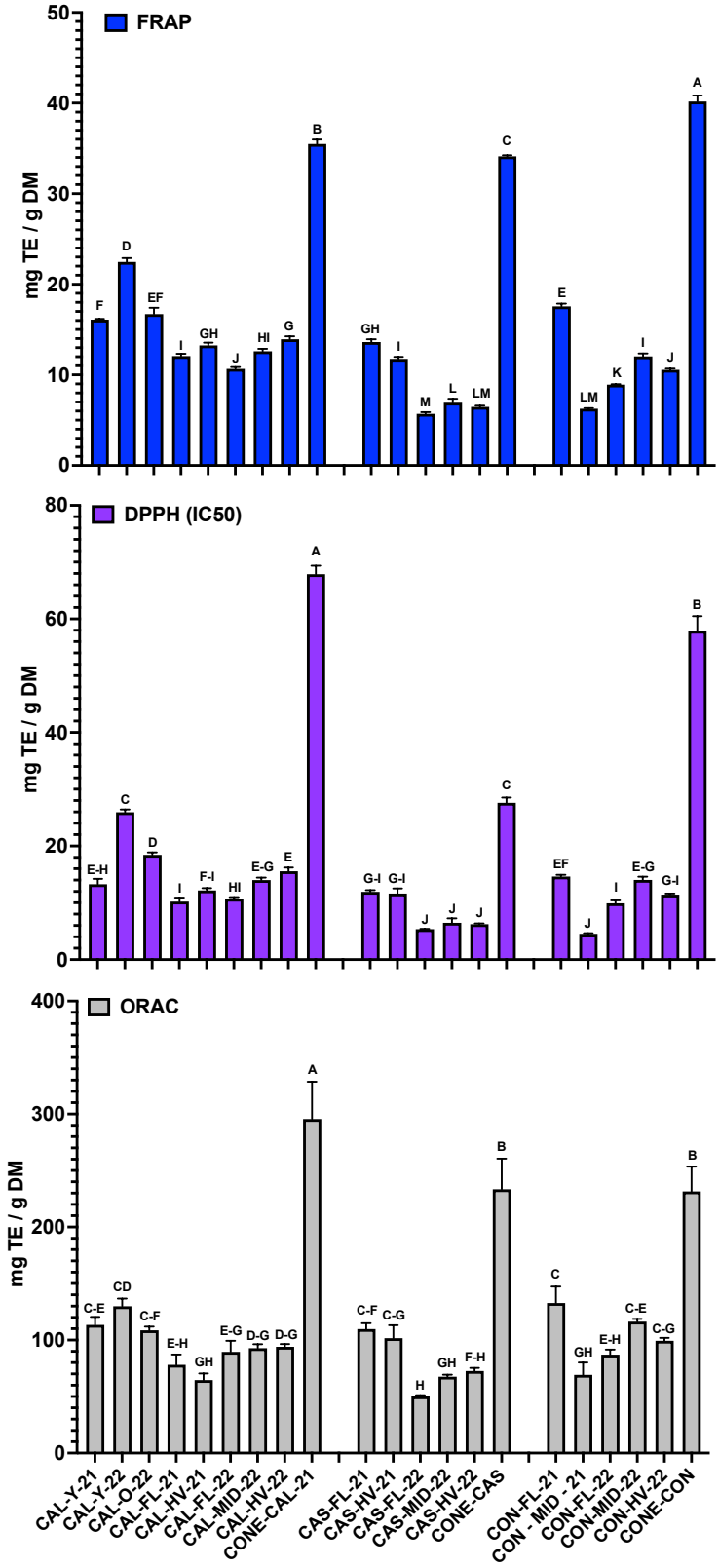


Figure 7.1: FRAP, DPPH (IC50) and ORAC antioxidant activities of hop leaf and cone extracts expressed as Trolox equivalents (TE).

Data represent the mean ± standard deviation of triplicate extractions (n=3). Letters represent ANOVA post-hoc groupings ($P < 0.05$).

CAL-Calypso. **CAS**-Cascade. **CON**-Contessa. **FL**-Flower stage. **MID**-Middle stage. **HV**-Harvest stage. **Y**-Young leaves. **O**-Old leaves. **21**-2021 crop year. **22**-2022 crop year. **CONE**-Hop cone.

The antioxidant activity of hop cone extracts was significantly higher than that of hop leaf extracts for all antioxidant assays employed in this study. Cone extract antioxidant activity ranged from 27.6-67.9 mg TE/g DM (DPPH), 34.1-40.2 mg TE/g DM (FRAP) and 231.56-295.58 mg TE/g DM (ORAC) whilst leaf extract activity ranged from 4.5-25.9 mg TE/g DM (DPPH), 5.7-22.5 mg/g DM (FRAP) and 50.1-132.8 mg/g DM (ORAC) depending on variety, stage of development and crop year. These findings are consistent with those reported by Stanius et al. (2022) and Abram et al. (2015), who also observed higher DPPH RSA (%) in hop cone extracts compared to their respective leaf extracts. However, Stanius et al. (2022) also found that the QUENCHER DPPH antioxidant activity was more comparable between the materials. The DPPH QUENCHER assay measures the solid parts of plant material, not just the extract, which suggests that hop leaves may have a higher proportion of 'bound' antioxidants.

The antioxidant activity of leaf extracts varied depending on variety, stage of development and crop year (Figure 7.1). Pearson's correlation analysis was performed to evaluate the relationships between the different antioxidant assays for hop leaf extracts and the scatter plots are presented in Appendix 7, Figure 4. The results revealed significant positive correlations between all assays ($P<0.001$), although particularly between the FRAP and DPPH assays ($P<0.0001$, Pearson $r = 0.9407$). This is most likely due to their similar electron transfer based mechanism (Kowalczyk et al., 2013). Many studies have shown a strong correlation between phenolic content and antioxidant activity, however polyphenols differ in antioxidant activity depending on their structure (Yordi et al., 2012).

Chapter 6 highlighted that mono-, di- and tri- glycosylated derivatives of quercetin and kaempferol were the most abundant phenolics in hop leaves. These were 3-O-glycosides which are generally more common than 7', 3' and 4' glycosides in hops (Dušek et al., 2021) and other plant species (Belitz et al., 2008). Generally, glycosylation has been shown to decrease flavanol antioxidant activity, although this depends on the number, position and

type of sugar moiety (Heim et al., 2002). Whilst glycosylation has not been reported to enhance antioxidant activity, it can enhance bioavailability (Hollman et al., 1999).

In this chapter antioxidant data was correlated with compositional data from Chapter 6 to see how closely the extracts' antioxidant activities could be predicted based upon their composition. Pearson's correlation analysis was performed to assess the relationship between antioxidant activity of hop leaf extracts (Figure 7.1) and their phenolic composition, as characterised in Chapter 6. Correlation analysis has been routinely used by researchers to identify key antioxidants in plant extracts (Ali et al., 2021). Given the significant differences in antioxidant activity and phenolic profile, hop cones were excluded from the analysis. Figure 7.2 presents a heat map illustrating the correlations between antioxidant assays and phenolic compound abundance, with rows and columns ordered according to AHC. Phenolic variables were grouped into 4 main clusters (C1-C4). Variables within C1 and C2 exhibited low correlations across all antioxidant techniques, with those in C2 showing particularly low correlations. In contrast, variables in C3 and C4 displayed high correlations, with those in C4 showing particularly high correlations. The phenolics variables generally grouped according to their structure, likely due to impact of phenolic structure on antioxidant activity (Yordi et al., 2012). Flavonol glycosides were grouped primarily by flavonol type, with quercetin glycosides generally grouped into C4 due to high correlations with electron transfer (ET) based antioxidant assays. In contrast the most abundant kaempferol glycosides in Cascade and Contessa hop leaves such as K-RUT and K-MG, K-GLUC and Clitorin (Chapter 6) were generally grouped into C1 and C2 corresponding to their lower correlations with all antioxidant assays.

Chlorogenic acids primarily clustered according to their hydroxycinnamic acid. Feruloylquinic acids grouped in C1 and C2 with low correlations to ET based techniques, whilst coumaroylquinic and caffeoylquinic acids were generally grouped in C3 and C4. Flavanols and

procyanidins were mostly grouped in C3 and C4 due to high correlations to all antioxidant techniques, particularly catechin, epicatechin and proanthocyanidin content (PB3E). Bitter resins were grouped in C1 and C2 whilst prenylflavonoids were more variable with desmethylxanthohumol, xanthohumol and 6-PN grouped in C3 and C4 whilst isoxanthohumol and prenylflavonone A were grouped into C1 and C2.

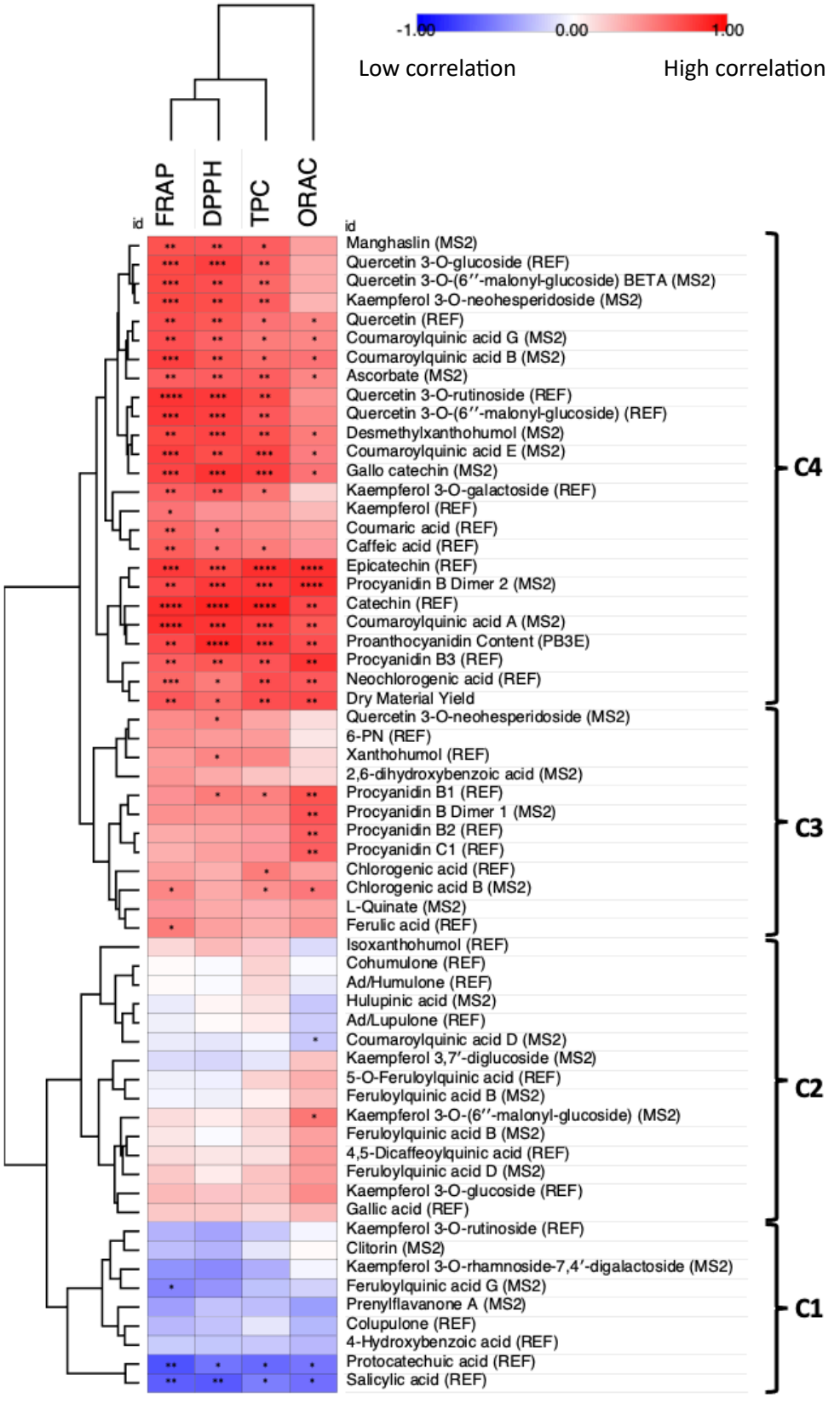


Figure 7.2: Heat map depicting Pearson's correlation coefficients (-1 – 1) between antioxidant activity and phenolic variables with columns and rows ordered by AHC (Euclidean distance).

Correlation significance levels are denoted as follows: * P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001.

This indicates that the most abundant phenolics in hop leaves, particularly the 3-O glycosides of kaempferol may have lower antioxidant capacity than other hop leaf phenolics. The high correlations between B-type procyanidins and antioxidant activity could also explain the significantly higher antioxidant activity of cone extracts (Figure 7.1), as they are particularly rich in these compounds, as outlined in Chapter 6. However, there are limitations to correlating antioxidant properties to phenolic composition, particularly when phenolic compounds with differing antioxidant properties also show high correlations to each other. To further explore the causal relationship between compound concentration and antioxidant activities, phenolic standards were evaluated individually for antioxidant activity.

7.4.2-Antioxidant Activities of Purified Phenolic Compounds

Phenolic standards for antioxidant analysis were selected based on their high concentrations in hop extracts, significant correlations to antioxidant activity (Figure 7.2) and availability as standards. Chlorogenic acids and flavonol glycosides were evaluated due to high concentrations in leaf extracts, whilst respective flavonol aglycones were assessed to determine the potential of using hydrolytic treatments to enhance the antioxidant activity of hop leaf extracts. B-type procyanidins were evaluated due to significant high correlations to antioxidant activity, whilst prenylflavonols were included for comparison as they are found in high concentrations in hop cones. Authentic phenolic standards were analysed for their DPPH, FRAP and ORAC activities as well as TPC. Trolox and caffeic acid equivalents on a weight and molar basis are presented in Appendix 7, Table 1 and 2 respectively for all compounds evaluated. Antioxidant differences were analysed using PCA, with the PCA plot presented in Figure 7.3.

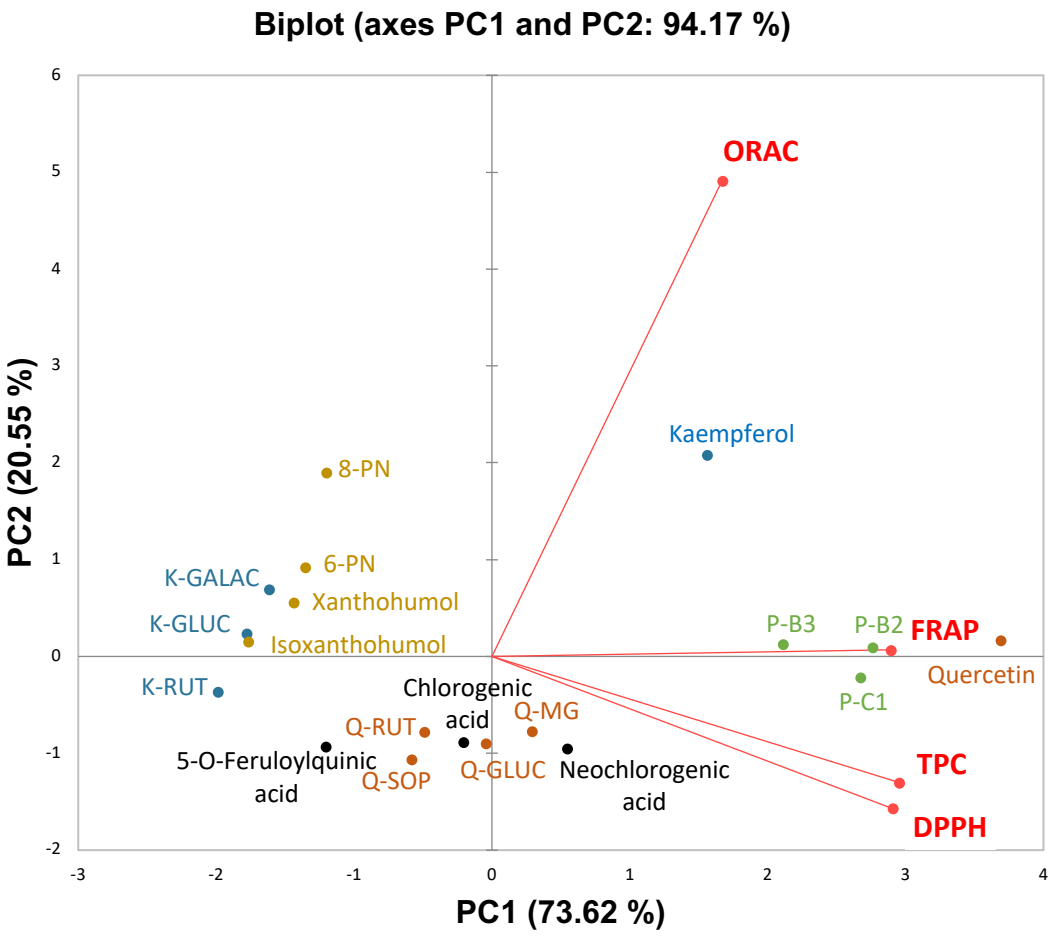


Figure 7.3: PCA plot of phenolic compound standards based on DPPH, FRAP and ORAC antioxidant activities as well as TPC. Antioxidant activity Trolox equivalents (mg TE/g compound) used for this analysis are provided in Appendix 7, Table 1. Colour-coded: kaempferol and its glycosides (blue), quercetin and its glycosides (brown), chlorogenic acids (black), prenylflavonoids (yellow) and procyanidins (green).

The first two principal components (PC1 and PC2) explained 94.17% of the variability. PC1 accounting for 73.6% of variance, was strongly positively correlated with TPC (0.953) and ET based antioxidant assays FRAP (0.933) and DPPH (0.937). PC2 accounting for 20.5% of variance was strongly correlated to ORAC (0.837). Figure 7.3 shows the antioxidant activity of phenolic standards generally grouped according to structural similarities, as observed in the correlation analysis (Figure 7.2). Kaempferol glycosides and prenylflavonoids grouped along PC1 due to very low ET antioxidant activity but separated along PC2 due to differences in ORAC activity. The ORAC activity of prenylflavonoids was in the order of: 8-PN > 6-PN >

xanthohumol > isoxanthohumol, which was consistent with findings by Van Hoyweghen et al. (2010). Quercetin glycosides and chlorogenic acids grouped along PC2 due to lower ORAC activity but separated along PC1 with Q-MG and neochlorogenic acid higher in ET based antioxidant activity. This aligns with Figure 7.2 where correlations between quercetin glycosides and ORAC activity were lower than those to ET based antioxidant assays. Procyanidins grouped with quercetin due to high ET antioxidant activity, whilst kaempferol was separated due to its lower ET activity but highest ORAC activity among all compounds. Flavonol glycosides had less positive loadings on both PC1 and PC2 compared to their respective aglycone. To investigate if glycoside hydrolysis could be an effective technique to improve antioxidant activity of these compounds, they were compared on a molar basis to their respective aglycones as is presented in Figure 7.4.

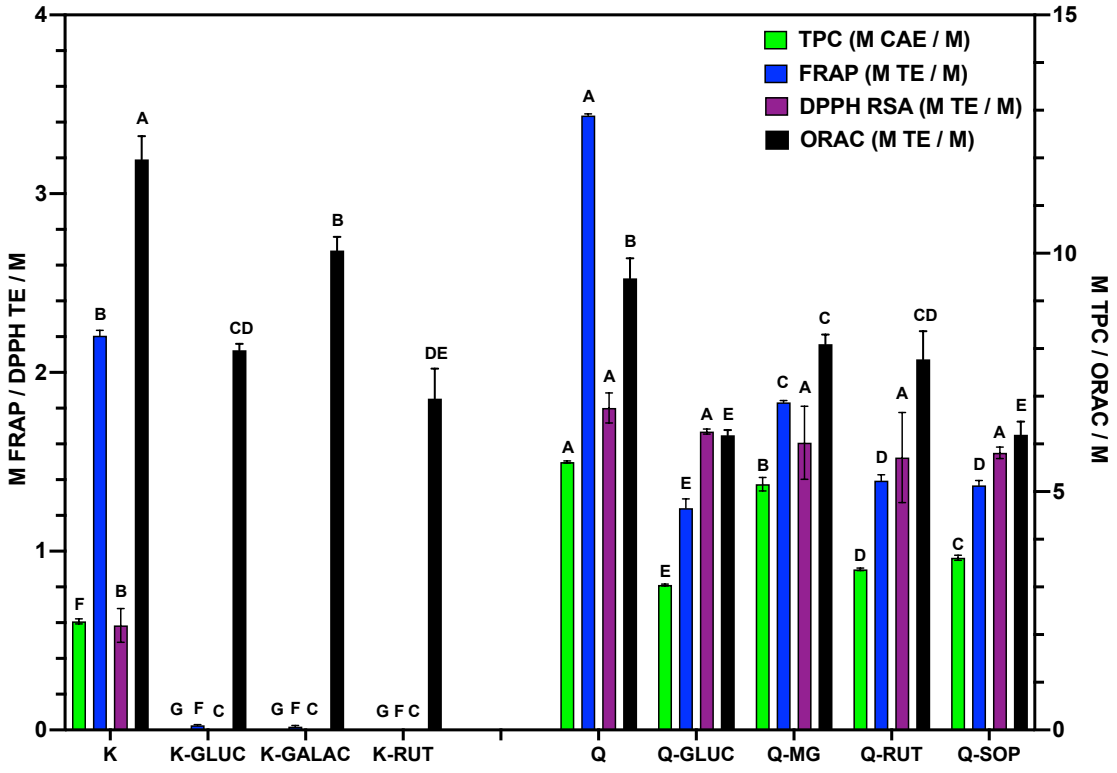


Figure 7.4: Antioxidant activities and TPC of quercetin, kaempferol and their respective glycosides presented on a molar basis.

Data represent the mean ± standard deviation of triplicate analytical measurements. Letters represent ANOVA post-hoc groupings ($P < 0.05$). **TE**-Trolox equivalent. **CAE**-Caffeic acid equivalents.

The antioxidant activities of flavonol glycosides was found to be significantly lower than for respective aglycone on a molar basis, except for the specific case of quercetin glycoside and DPPH activity. Quercetin and its glycosides exhibited higher activity in ET based techniques (DPPH, FRAP and TPC), whilst kaempferol exhibited higher ORAC activity. Q-MG was the most potent quercetin glycoside according to all methods (other than DPPH) with Trolox and caffeic acid molar equivalents of 5.15 (TPC) 1.83 (FRAP) 6.18 (ORAC) and 1.61 (DPPH). Spreng and Hofmann (2018) also found that on a molar basis Q-MG exhibited greater ORAC and DPPH activity than Q-GLUC and it was highlighted as a key antioxidant in beer. However they also reported that K-MG had lower ORAC activity than K-GLUC on a molar basis, suggesting that the effect of malonylation on antioxidant activity may depend on the specific flavonol (Spreng and Hofmann, 2018). Differences in FRAP activity between aglycone and glycoside were greater than differences for ORAC activity indicating that glycosylation negatively impacts the ET antioxidant mechanism more so than the hydrogen atom transfer (HAT). However, this was not the case for quercetin glycoside DPPH activity which exhibited no statistically significant differences. This was unexpected as the DPPH assay has been shown to correlate highly with the FRAP assay (Dudonne et al., 2009).

Notably, all kaempferol glycosides exhibited negligible ET activity whilst kaempferol activity was significantly higher (FRAP-2.21 TE, FRAP-0.52, TPC-2.26 CAE). Negligible ET based antioxidant activity has been found for various kaempferol glycosides in the literature including kaempferol 3-O-(6''-malonyl-glucoside) (Katsube et al., 2009), which was highly abundant in Contessa hop leaves. Wang et al. (2018a) and Braca et al. (2003) also reported significantly lower antioxidant activity for kaempferol 3-O-glycosides, however found that 7-O-glycosides exhibited similar DPPH reduction and ABTS activity, indicating that position as well as flavonol may impact glycoside antioxidant activity. It is hypothesised this is due to steric hindrance of 3-O-glycosylation forcing the B-ring not to be coplanar with the A- and C-ring thereby weakening the molecules' conjugation system (Xiao et al., 2021). Park et al.

(2006) also found negligible DPPH activity for two kaempferol tri-glycosides but that an enzyme combination of β-galactosidase and hesperidinase was effective for glycoside hydrolysis which resulted in a kaempferol purity of 95% and significantly higher DPPH reduction.

This indicates that plant materials rich in kaempferol 3-O-glycosides may be the most promising candidates for hydrolytic treatment for improved antioxidant activity. Hop leaves have been shown to be a rich source of flavonol glycosides but with glycoside structure depending predominantly on variety. It is hypothesised that Cascade and Contessa hop leaves rich in kaempferol 3-O-rutinoside, clitorin and Kaempferol 3-O-(6''-malonyl-glucoside) are more suited to enzymatic glycoside cleavage for improved antioxidant activity compared to Calypso leaves which are predominantly made up of quercetin 3-O-glycosides.

7.4.3 Snailase hydrolysis of hop leaf extracts and flavonol glycosides

7.4.3.1 Phenolic content

For enzyme hydrolysis trials, three leaf samples were evaluated; CAS-FL-21, S. Passion-HV-21 and S. Aroma-HV-21. These samples were selected for snailase treatment due to their high but varied flavonol glycoside contents to evaluate affinity of snailase towards these different structures. Leaf extracts were purified using C18 SPE, treated with snailase and analysed for phenolic content using LC-qTOF-MS/MS. It has been demonstrated by authors such as Kornpointner et al. (2022) and Chen et al. (2024) that purification is not essential for snailase treatment, however purification was performed in this study to limit the possibility of hop leaf extract components interacting negatively with snailase activity. Table 7.3 presents the concentrations of flavonols and their glycosides in the snailase-treated and control hop leaf extracts. Semi-quantitation was performed using standard curves from structurally similar standards where available.

Table 7.3: Flavonol glycoside hydrolysis rates (%) and concentrations in treated and control leaf extracts (mg/g).

	Cascade			S. Passion			S. Aroma		
	Control	**Snailase Treated**	**Hydrolysis**	**Control**	**Snailase Treated**	**Hydrolysis**	**Control**	**Snailase Treated**	**Hydrolysis**
Q-GLUC (REF)	1.42 ± 0.09	0.11 ± 0.04	92.54	13.61 ± 0.35	0.13 ± 0.02	99.02	15.29 ± 0.50	0.15 ± 0.11	99.06
K-GLUC (REF)	13.53 ± 0.32	0.34 ± 0.03	97.56	26.92 ± 0.72	0.27 ± 0.02	99.01	31.70 ± 0.87	0.40 ± 0.04	98.74
Q-RUT (REF)	20.47 ± 0.10	< LOQ	100.00	14.39 ± 0.40	< LOQ	100.00	10.14 ± 0.29	< LOQ	100.00
K-RUT (REF)	400.56 ± 6.25	< LOQ	100.00	69.14 ± 1.18	< LOQ	100.00	47.16 ± 0.80	< LOQ	100.00
Q-M GLUC (REF)	2.90 ± 0.03	< LOQ	100.00	12.91 ± 0.45	< LOQ	100.00	17.93 ± 0.19	< LOQ	100.00
K-MG (MS2) (Q-MG equivalent)	92.02 ± 0.77	< LOQ	100.00	79.68 ± 1.16	< LOQ	100.00	111.43 ± 2.85	< LOQ	100.00
Q-NEO (MS2) (Q-RUT equivalent)	1.40 ± 0.03	< LOQ	100.00	2.13 ± 0.14	< LOQ	100.00	3.25 ± 0.17	< LOQ	100.00
K-NEO (MS2) (K-RUT equivalent)	1.62 ± 0.10	< LOQ	100.00	< LOQ	< LOQ	-	< LOQ	< LOQ	-
Q-SOP (REF)	1.53 ± 0.07	< LOQ	100.00	0.57 ± 0.10	< LOQ	100.00	0.36 ± 0.03	< LOQ	100.00
Manghaslin (MS2) (Q-RUT equivalent)	4.39 ± 0.14	< LOQ	100.00	2.17 ± 0.02	< LOQ	100.00	2.68 ± 0.10	< LOQ	100.00
Clitorin (MS2) (K-RUT equivalent)	55.08 ± 0.92	< LOQ	100.00	4.71 ± 0.14	< LOQ	100.00	7.30 ± 0.10	< LOQ	100.00
Total Kaempferol glycosides	562.82 ± 8.36	0.34 ± 0.03	99.86	180.45 ± 3.20	0.27 ± 0.16	99.59	197.59 ± 4.62	0.40 ± 0.31	99.53
Total Quercetin Glycosides	32.10 ± 0.46	0.11 ± 0.04	99.64	45.79 ± 1.45	0.13 ± 0.02	99.71	49.66 ± 1.27	0.15 ± 0.11	99.71
Total Glycosides	594.92 ± 8.82	0.45 ± 0.25	99.85	226.25 ± 4.66	0.40 ± 0.18	99.61	247.25 ± 5.89	0.55 ± 0.41	99.56
Kaempferol	0.16 ± 0.00	152.54 ± 2.70		0.25 ± 0.03	66.73 ± 2.55		0.28 ± 0.04	77.55 ± 3.62	
Quercetin	0.12 ± 0.05	13.27 ± 0.69		0.28 ± 0.05	23.46 ± 1.49		0.30 ± 0.12	27.11 ± 1.91	
Total Aglycones	0.28 ± 0.05	165.81 ± 3.39		0.53 ± 0.08	90.19 ± 4.04		0.58 ± 0.16	104.66 ± 5.52	

Hydrolysis (%): Control flavonol glycoside extract concentration/sum of flavonol glycoside concentrations in treated and control extracts * 100.

REF-Phenolics identified with a reference standard.

MS2-Phenolics identified using MS/MS fragmentation patterns.

7.4.3.1.1-Flavonols

The dominant glycosides for all control extracts were 3-O mono- and di- glycosides of kaempferol and quercetin with tri- glycosides such as clitorin and manghaslin present at lower levels. This is in accordance with analysis of their respective crude ethanolic extracts detailed in Chapters 4 and 6. The profile of flavonol glycosides in Cascade control extract was distinguishable from that of S. Aroma and S. Passion control extracts which were more similar to one another. This is most likely attributed to the closer genetic relationship and shared location of cultivation (South African) of S. Aroma and S. Passion both of which are recognised factors influencing phenolic composition (Abram et al., 2015). Cascade control extract had a much higher concentration of all flavonol di- and tri- glycosides in particular kaempferol 3-O-rutinoside (400.56 mg/kg) compared to S. Aroma (47.16 mg/g) and S. Passion (69.14 mg/g). S. Aroma and S. Passion exhibited higher concentrations of quercetin glycosides but these were still substantially lower than kaempferol glycosides for all varieties. The aglycones kaempferol and quercetin were detected in all control extracts but at very low concentrations (> 0.6 mg total aglycones/g).

In snailase treated extracts, flavonol glycoside concentrations were very low for all varieties (<0.5 mg/g) with only Q-GLUC and K-GLUC found in the range of quantitation. This represented conversion rates of 92.5-99.1% for Q-GLUC, 97.6-99.0% for K-GLUC and approximately 100% for all other glycosides. Chen et al. (2024) found similarly high snailase hydrolysis rates for structurally similar flavonol glycosides using comparable incubation conditions. The slightly lower theoretical hydrolysis rates for mono-glucosides could be a result of lower snailase affinity for that substrate, but it seems more likely that these are intermediate products of the hydrolysis of flavonol rutinosides, which were present at much higher concentrations. Wang et al. (2011) demonstrated that the products of Q-RUT snailase hydrolysis were quercetin and Q-GLUC indicating that Q-GLUC is an intermediate product of Q-RUT hydrolysis to quercetin.

Glycoside hydrolysis resulted in a significant increase in the concentrations of kaempferol and quercetin for all extracts, particularly for Cascade which had the highest total aglycone concentration. Kaempferol was the dominant aglycone for all varieties at 91.99 % (Cascade), 73.98 % (S. Passion) and 74.10 % (S. Aroma) of total aglycones. As expected, the concentrations and ratios of aglycones in the snailase-treated extracts corresponded to those of flavonol glycoside in the control extracts. Table 7.4 presents the predicted aglycone yield on a molar basis for the hydrolysis of each flavonol glycoside, considering the concentrations and hydrolysis (%) presented in Table 7.3.

Table 7.4: Aglycone yields for snailase treated hop leaf extracts.

Compound	g/mol	Predicted aglycone yield (mg/g)		
		Cascade	S. Passion	S. Aroma
Q-GLUC (REF)	464.38	0.85	8.77	9.86
K-GLUC (REF)	448.38	8.42	17.01	19.98
Q-RUT (REF)	610.52	10.13	7.13	5.02
K-RUT (REF)	594.52	192.85	33.29	22.71
Q-M GLUC (REF)	550.42	1.59	7.09	9.85
K-M GLUC (MS2)	534.43	49.06	42.42	59.40
Q-NEO (MS2)	610.52	0.69	1.06	1.61
K-NEO (MS2)	594.52	0.78	0.00	0.00
Q-SOP (REF)	626.55	0.74	0.28	0.17
Manghaslin (MS2)	756.66	1.75	0.87	1.07
Clitorin (MS2)	740.66	21.28	1.82	2.82
Predicted Q concentration		15.76	25.19	27.58
Observed Q concentration		13.27	23.46	27.11
Q yield (%)		84.19	93.16	98.29
Predicted K concentration		272.39	94.54	104.90
Observed K concentration		152.54	66.73	77.55
K yield (%)		56.00	70.58	73.93
Predicted aglycone concentration		288.15	119.73	132.48
Observed aglycone concentration		165.81	90.19	104.66
Aglycone yield (%)		57.54	75.33	79.00

Predicted aglycone: (Control flavonol glycoside (FG) concentration – Treated FG concentration) / (FG MW / aglycone MW).

Predicted Q concentration: Sum of all predicted quercetin glycoside aglycone yields.

Observed Q concentration: Quercetin concentration in the treated extracts.

Q yield: (Predicted Q concentration / Observed Q concentration) * 100.

Predicted K concentration: Sum of all predicted kaempferol glycoside aglycone yields.

Observed K concentration: Kaempferol concentration in treated extracts.

K yield: (Predicted K concentration / Observed K concentration) * 100.

Predicted aglycone concentration: Sum of predicted Q and K concentrations.

Observed aglycone concentration: Sum of observed Q and K concentrations.

Aglycone yield: (Predicted aglycone concentration / Observed aglycone concentration) * 100.

REF-Phenolics identified with a reference standard.

MS2-Phenolics identified using MS/MS fragmentation patterns.

Quercetin yields (84.2-98.3%) were consistently higher than kaempferol yields (56.00-73.93%) across all varieties. The underlying cause of this is not clear but may involve aglycone degradation during incubation, formation of undetected hydrolysis products or complexation with the precipitate formed after the addition of ethanol. Wang et al. (2012) observed that quercetin concentrations decreased over incubation in snailase-treated rutin samples whereas a comparison enzyme (cellulase-T2440) maintained more stable quercetin levels. This effect may stem from the crude composition of snailase which contains glycoside releasing cellulases, but also other enzymes that may degrade kaempferol more readily than quercetin upon glycoside hydrolysis. It is suggested that future trials evaluate snailase treatments at varying concentrations and incubation times to optimise the hydrolysis of glycosides whilst limiting degradation of aglycones. However, it is worth noting no significant differences were found between control and snailase treated extracts for catechin, epicatechin or xanthohumol (Appendix 7, Table 3).

7.4.3.1.2 Phenolic acids

Phenolic acids were also found in significantly higher levels for snailase treated extracts compared to controls. Figures 7.5 and 7.6 present the levels of quantified phenolic acids and those measured by IS normalised abundance respectively. Coumaric, protocatechuic, hydroxybenzoic and caffeic acids were all < LOD for control samples whilst gallic and quinic acid were found in very low levels. Treated extracts had significantly higher levels of all phenolic acids than respective control samples however these didn't exceed 800 mg/kg for each compound. Phenolic acids can be present in free form but also bound to sugars and quinic acid to form high molecular weight hydrolysable tannins, and low molecular weight chlorogenic acids and phenolic acid glycosides (Ribeiro, 2015). Li et al. (2020) previously demonstrated that snailase can hydrolyse protocatechuic, p-hydroxybenzoic, vanillic and p-coumaric acid glycosides releasing the free phenolic acid. Like flavonols, free phenolic acids have been found to exhibit higher antioxidant activities than their respective glycosides. The

higher levels of protocatechuic acid and gallic acid in snailase treated extracts compared to control indicates the presence of glycosidically bound forms in the hop leaves evaluated in this study. However, these are likely present at low levels as free phenolic acids concentrations quantified in treated extracts were < 1.5 mg total/g.

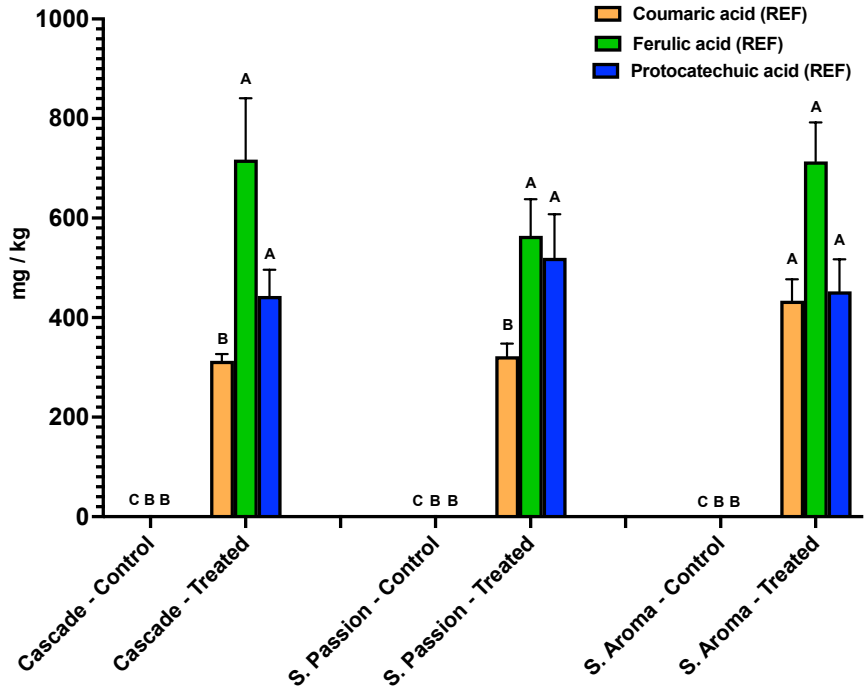


Figure 7.5: Concentrations of coumaric, ferulic and protocatechuic acid for snailase treated and control leaf extracts.

Data represent the mean ± standard deviation of duplicate treatments (n=2). Letters represent ANOVA post-hoc groupings ($p<0.05$).

REF-Phenolics identified with a reference standard.

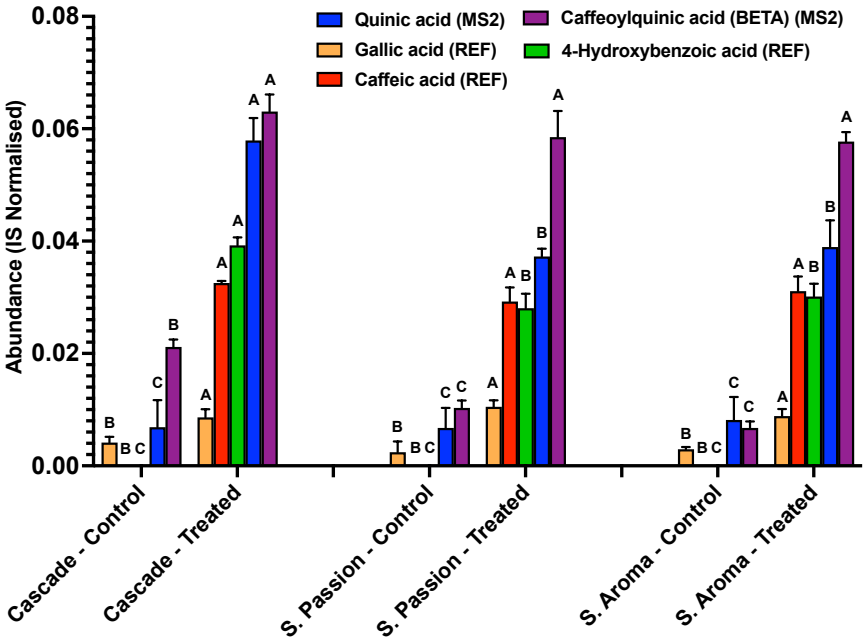


Figure 7.6: Normalised abundance of quinic, caffeic, gallic, caffeoylquinic (BETA) and 4-hydroxybenzoic acid for snailase treated and control leaf extracts.

Data represent the mean ± standard deviation of duplicate treatments (n=2). Letters represent ANOVA post-hoc groupings ($p<0.05$).

MS2-Phenolics identified using MS/MS fragmentation patterns. **REF**-Phenolics identified with a reference standard.

7.4.3.2-Antioxidant analysis of Snailase treated materials

Snailase treated and control extracts were evaluated for antioxidant activity using DPPH, FRAP and ORAC assays to evaluate the impact of glycoside hydrolysis on ET and HAT antioxidant activities. Incubation controls were included for leaf extracts to ensure that any observed antioxidant differences were related to snailase treatment rather than incubation conditions.

The antioxidant activities of snailase treated and control phenolic standards are presented in Figure 7.7. Kaempferol glycosides had the lowest DPPH and FRAP control activity but demonstrated the greatest increases on Snailase treatment for these assays. Quercetin glycosides had higher DPPH and FRAP control activities but demonstrated more moderate increases on treatment for FRAP and DPPH. This difference can be attributed to the greater hindrance of 3-O glycosylation on ET antioxidant properties of kaempferol over quercetin as proposed in section 7.4.2.

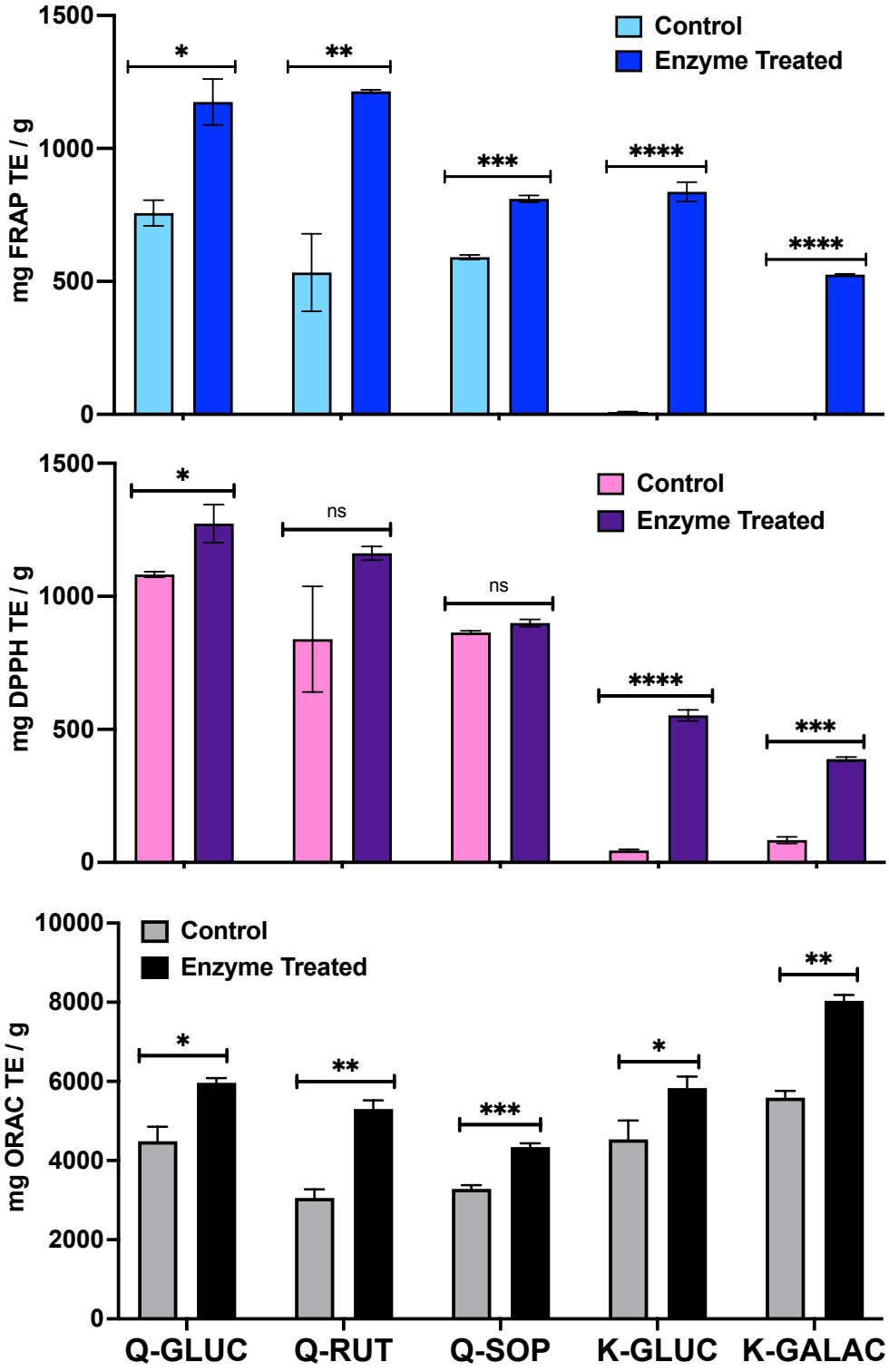


Figure 7.7: Antioxidant activity (FRAP, DPPH and ORAC) of snailase treated and control kaempferol and quercetin glycoside phenolic standards. Data expressed as Trolox equivalents (mg TE/g).

Data represent the mean ± standard deviation of duplicate treatments (n=2).

T-test significance levels are denoted as follows: * P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001.

The antioxidant activities of snailase-treated and control hop leaf extracts are presented in Figure 7.8. The snailase-treated extracts generally exhibited significantly higher antioxidant activity than control extracts, with variations depending on hop leaf variety and the antioxidant assay. The increase in antioxidant activity upon snailase treatment followed the order: Cascade > S. Passion > S. Aroma, with the most substantial differences observed in the FRAP assay. No significant differences were found between incubation control and control extracts, indicating that the incubation conditions adopted in this study did not impact antioxidant activity.

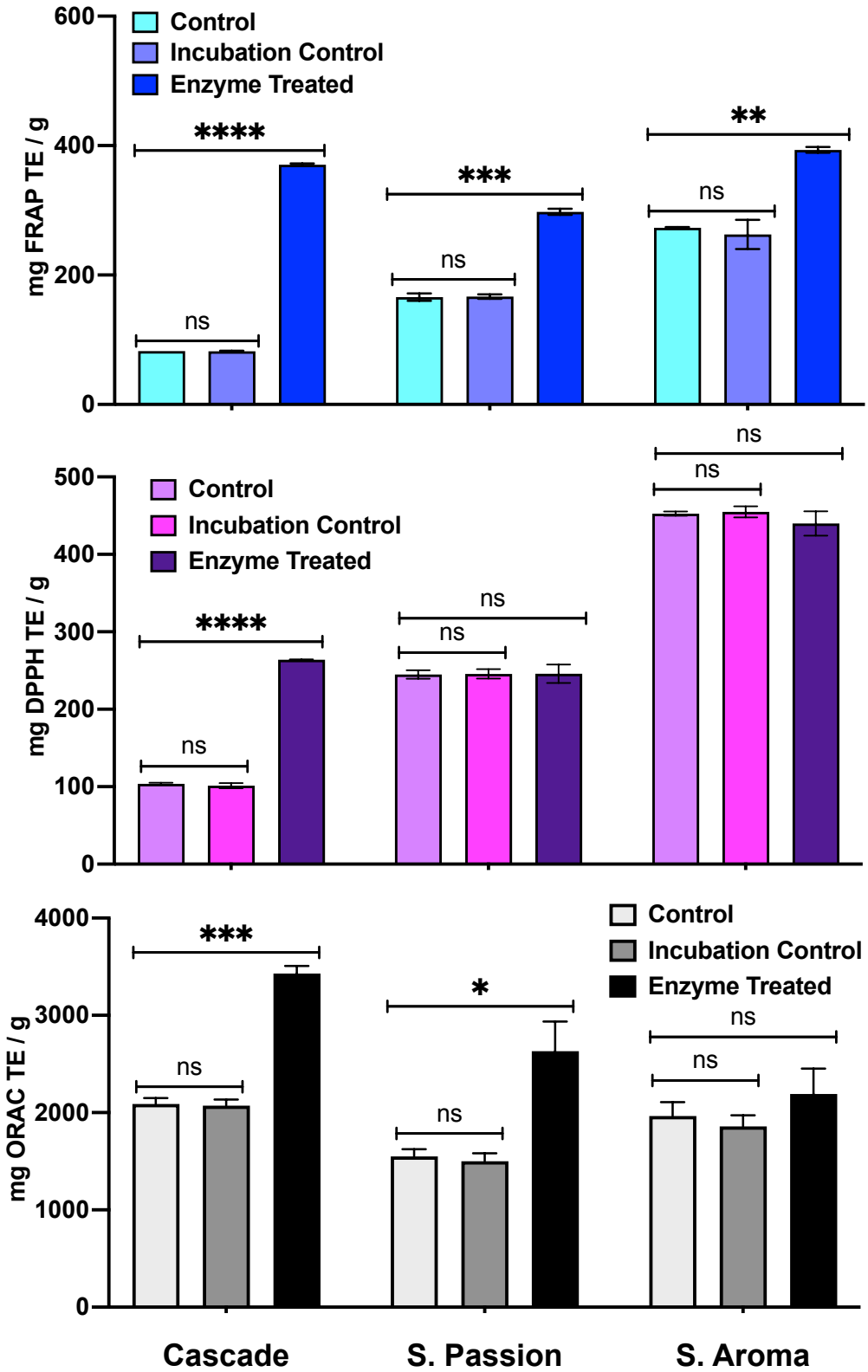


Figure 7.8: Antioxidant activity (FRAP, DPPH and ORAC) of snailase treated and control Cascade, S. Passion and S. Aroma purified hop leaf extracts. Data expressed as Trolox equivalents (mg TE/g).

Data represent the mean ± standard deviation of duplicate treatments (n=2).

T-test significance levels are denoted as follows: * P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001.

The antioxidant activities of treated and control leaf extracts strongly correlated with their flavonol glycoside contents and their antioxidant activity relative to respective aglycones, as detailed in Figure 7.4 and Tables 7.3 & 7.4. For the DPPH and FRAP assays, Cascade exhibited the lowest control antioxidant activity, but also the largest fold increases upon snailase treatment with 4.51-fold (FRAP) and 2.54-fold (DPPH) increases. S. Passion and S. Aroma had higher control antioxidant activity but displayed smaller fold increases upon snailase treatment at 1.7-fold and 1.44-fold (FRAP) respectively, with no significant differences observed for DPPH. This is likely related to the higher content of kaempferol 3-O-glycosides for Cascade, which exhibited negligible ET antioxidant activity (Appendix 7-Table 1) and the higher content of quercetin 3-O-glycosides in S. Aroma and S. Passion, which exhibited higher ET antioxidant activity but with smaller differences compared to quercetin.

For the ORAC assay, similar trends were observed, with the greatest activity increases upon snailase treatment seen for Cascade (1.64-fold) and S. Passion (1.70-fold), whilst no significant difference was observed for S. Aroma. Notably, Cascade exhibited the highest ORAC activity among the three varieties, both in the control (2087.62 mg TE/g) and treated (3429.59 mg TE/g) samples. The higher ORAC activity in treated Cascade between varieties can be attributed to its high kaempferol content, which was the most potent antioxidant analysed in the ORAC assay (Appendix 7, Table 1). The higher activity in control Cascade compared to the other varieties is likely due to its higher kaempferol glycosides content which were generally more potent antioxidants in the ORAC assay compared to quercetin glycosides (Appendix 7, Table 1).

To illustrate groupings of standards and extracts based on antioxidant activity differences between control and treated extracts, fold changes in activity (ranging from 1 to ≥5) are presented as a heat map in Figure 7.9, with rows and columns ordered according to AHC. Trolox equivalents were not calculated for snailase-treated K-RUT and Q-MG standards due

to the unknown sample weight (<0.2 mg) however they were evaluated on a fold increase basis. Cascade extract grouped more closely to kaempferol glycosides, which is attributed to greater fold increases in FRAP and DPPH activities whereas S. Aroma and S. Passion group more closely with quercetin glycosides, which is attributed to lower fold increases in these assays. Figure 7.9 clearly highlights that flavonol type, specifically whether a glycoside is quercetin or kaempferol based, had the greatest impact on antioxidant activity differences of flavonol glycosides following hydrolytic treatment.

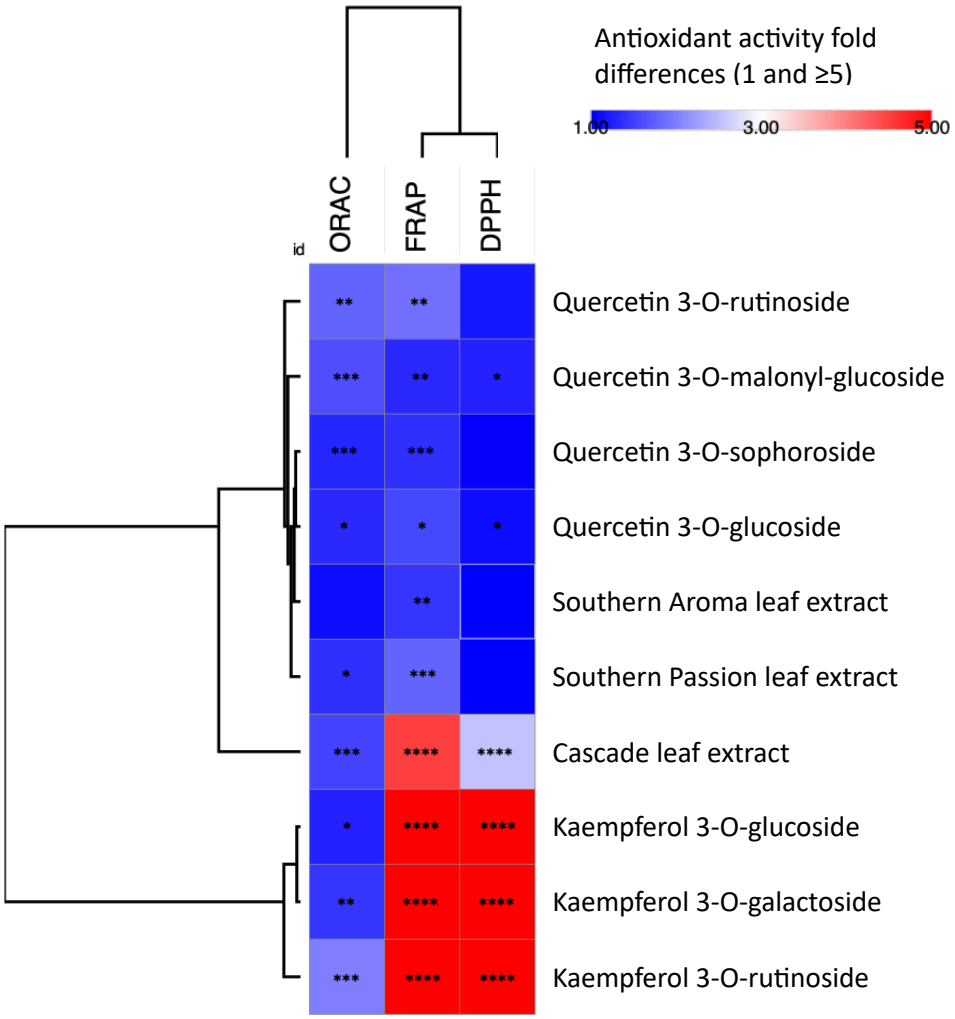


Figure 7.9: Fold differences in antioxidant activity between 1 and ≥5 between snailase treated and control phenolic standards and purified hop leaf extracts. Rows and columns ordered according to AHC analysis (Euclidean distance).

T-test significance levels are denoted as follows: * P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001.

7.4.3.3-Antioxidant activity comparison: Hop leaves vs commercial extracts

To benchmark the antioxidant activity of treated leaf extracts, comparisons were made with commercial extracts and PAD950 SPE purified hop fractions analysed in Chapter 5. Commercial extracts were predominantly derived from agri-food co-products including grape pomace (Grape OPC, Vitisol), and pine bark (Pineol). The DPPH and FRAP antioxidant activities of commercial extracts and purified hop fractions were higher for all comparison materials, except ProXantho (hydrophobic precipitate comprising xanthohumol and alpha acids). These extracts are predominantly made up of proanthocyanidins which exhibited comparable ET activity to kaempferol as analysed in this study (Appendix 6, Table 1). The lower ET activity observed in the treated leaf extracts can be attributed to the presence of hydrolysed sugars resulting from the cleavage of flavonol glycosides. Notably, the hydrolysis of kaempferol 3-O-rutinoside yields only 42.1% kaempferol with the remaining 57.9% comprised of glucose and rhamnose. Monosaccharides exhibit negligible antioxidant capacity (Hu et al., 2016), thereby limiting the antioxidant potential of the resulting extract. This suggests that to produce high antioxidant activity extracts, leaf extracts should undergo snailase treatment prior to SPE purification and that an adsorbent with poor monosaccharide affinity (such as C18) should be used.

Snailase treated hop leaf extracts were shown to have higher ORAC values compared to CyanthOx 50, Green Tea 38 and the hydrophobic hop residue extract but lower values than Vitisol, Pineol and Grape OPC. Snailase treated Cascade extract was comparable to the hop cone hydrophilic extract indicating that with hydrolytic treatment leaf phenolic extracts can be enhanced to match the antioxidant capacity of cone-derived hop co-products.

Chapter 8-Conclusions and Recommendations for Future Works

8.1-Conclusions

In this study, hop co-products from the harvesting and processing of hop cones were comprehensively evaluated for their phenolic content and antioxidant activity. The primary aim was to assess these co-products as a source of natural phenolics to produce extracts with high antioxidant capacity and thus potential added value for applications across various industry sectors. As such, green extraction techniques were adopted so that findings could potentially be scaled-up to produce non-toxic food grade extracts safe for the cosmetic, food and beverage sectors. Initially the extraction conditions were optimised using aqueous ethanolic solutions, which were then applied to a range of hop co-products and non-hop plant materials to compare the phenolic content and antioxidant activities of their extracts. Different purification techniques were then evaluated for the phenolic extracts, guided by maximising antioxidant activity and phenolic purity. The resulting fractions were benchmarked against well-established commercial phenolic extracts from different plant sources. Hydrolytic treatment was also evaluated to enhance the antioxidant capacity of hop leaf extracts.

This study has reported the following significant findings:

Chapter 3 aimed to optimise the extraction of phenolics and antioxidant activity from CO_2R-HERK using 'green' solvents and techniques. Aqueous ethanol was chosen as the extraction solvent due to its lower toxicity compared to conventional extraction solvents and demonstrated effectiveness in phenolic extraction across a range of plant materials. Enzyme-assisted extraction was also explored to assess the release of bound phenolics. A central composite design was employed to evaluate the effect of ethanol % of extraction solvent (30-90%), and solid: liquid ratio (100-150 mg/ml). Ethanol concentration of aqueous extraction solution was the most significant factor evaluated which influenced the extraction of

phenolics and resins from commercial hop CO_2 extraction residue (cv. Herkules) ($p<0.0001$). The optimal ethanol concentration for the extraction of total phenol content (TPC), proanthocyanidin content (PAC) and FRAP antioxidant activity was between 40-50% v/v (aq), whilst xanthohumol and alpha acids were best extracted at 65-75% v/v (aq). Ultimately, 50% ethanol v/v (aq) was determined to be the most effective concentration for the simultaneous extraction of TPC, PAC, xanthohumol, alpha acids and FRAP antioxidant activity. Solid: liquid ratio of the extraction did not have a significant impact on the extraction as evaluated by any of the assays, within the evaluated range of 100-150 mg/ml ($p > 0.05$). This indicates that higher solid: liquid ratios could be selected that are more viable for commercial-scale extraction due to lower solvent and processing costs.

A range of commercial enzyme preparations (comprising pectinases, cellulases and proteases) were evaluated at 1% and 0.1% DM. Supplier recommended incubation temperature and pH conditions were used with a 90 min aqueous enzyme incubation step prior to a 50% (v/v) ethanolic extraction. Whilst some enzyme treated extracts had a higher TPC compared to their incubation control, differences were small and not significant ($P>0.05$). This may be due to low levels of bound phenolic compounds in CO_2R-HERK, or the use of non-optimal incubation conditions. Higher incubation temperatures (60 °C) significantly decreased phenolic extraction ($P<0.05$), indicating that hop phenolics may degrade at elevated temperatures, making high optimal temperature enzyme preparations unsuitable for enhancing phenolic extraction from hop processing residues. Considering these findings, the extraction conditions considered to most effective, and therefore selected for use in subsequent trials were 50% ethanol concentration, a 100 mg/ml solid: liquid ratio and no enzyme additions.

In Chapter 4 the phenolic profiles of extracts of a range of hop co-products and non-hop materials were analysed and compared with one another. Hop materials analysed included

hop pellets, leaves and processing residues from T45 pelleting and CO_2 extraction of different varieties. Non-hop materials included those known for their high phenolic content, such as seabuckthorn pomace, cinnamon, Rooibos tea and various berry samples. Hop co-product extracts generally had higher phenolic contents, particularly proanthocyanidins, as well as higher antioxidant activities compared to the non-hop materials. The most abundant phenolic sub-groups identified in hop co-products were flavanols, B-type procyanidins, flavonol glycosides, prenylflavonols and chlorogenic acids. The composition of co-products was predominantly dependant on the co-product stream and variety. Prenylflavonols were predominant in CO_2 extraction residues, catechins and procyanidins were the main constituents in T45 pelleting residues whereas in hop leaves the quantitatively significant fractions were flavonol glycosides and chlorogenic acids.

Comparisons with published literature indicated that CO_2 extraction leads to a greater degree of phenolic enrichment in the residue compared to T45 pelleting, although this was not initially clear due to inconsistent sourcing of varieties of the various co-products. It was concluded that, in general hop CO_2 extraction residue is a more promising source of phenolic compounds compared to T45 processing residues, however this of course also depends on the phenolic composition of the initial cone. CO_2R-HERK was highlighted as a particularly promising material for the subsequent trial to purify and enrich extracts, due to the high prenylflavonoid content (9.58 mg total prenylflavonoids/g DM). Xanthohumol, the principal prenylflavonoid of CO_2R-HERK extract, has received attention for its potent hydroxyl and peroxyl radical scavenging activity (Van Hoyweghen et al., 2010), and anti-cancer properties (Girisa et al., 2021). Considering the significance of these properties, and the high material availability of CO_2R-HERK, with Herkules being the currently preferred variety for CO_2 extraction, it was selected for subsequent purification trials.

Purification of phenolic extracts is an important step to remove non desirable components whilst concentrating phenolic compounds to generate purified extracts with higher antioxidant activities. **Chapter 5** aimed to evaluate two SPE adsorbents (PVPP and PAD950), and three ultrafiltration membranes (UFX10, FS40, GR51) for the purification of CO_2R-HERK extract and compare the phenolic purity and antioxidant activities of the resulting fractions. PVPP and PAD950 were evaluated due to reported high affinities to prenylflavonols and procyanidins respectively, whilst ultrafiltration was evaluated due to its lower cost. For purification treatment ethanol was removed from CO_2R-HERK resulting in a hydrophobic precipitate.

The highest purity extract was produced by desorption of treated PAD950, a macroporous resin that adsorbs phenolics through hydrophobic interactions and hydrogen bonding. This fraction was mainly composed of catechins, B-type procyanidins and flavonol 3-O-glycosides. Ultrafiltration produced a retentate and permeate for each membrane and was found to be much less effective across a range of different membrane sizes evaluated between 10-100 kDa. It was thus concluded that purification of extracts using PAD950 SPE offers greater phenolic specificity as compared to molecular weight size exclusion techniques. PVPP was also investigated as a novel method for concentrating phenolics from hop extracts. Data were reported in the form of Langmuir isotherm modelling, demonstrating high adsorption capacities of PVPP for hop phenolics from both E0 and E50 feed solutions. However, hurdles remain to be overcome for the efficient desorption of phenolics from PVPP using 'green' solvents. Overall, it was highlighted that phenolics from CO_2R-HERK can be purified using 'green' techniques to generate extracts with comparable antioxidant activities to those of commercial extracts; solid phase extraction using a PAD950 phase offered the best enrichment.

Chapter 6-Hop leaves represent an under-utilised source of biomass with potential for valorisation as a source of bioactive phenolic compounds. In **Chapter 4** hop leaves were shown to have the most distinct phenolic profile among the co-products evaluated, with notably higher levels of flavonol glycosides and chlorogenic acids. This study aimed to evaluate the phenolic composition of hop leaf and cone extracts for three commercially significant varieties. Leaf materials was sourced from Yakima, the highest acreage growing location, over two crop year and three stages of development.

LC-MS/MS in silico fragmentation identified a diverse range of phenolics in the hop leaf extracts, the most abundant of which were quercetin and kaempferol 3-O mono, di- and tri-glycosides and chlorogenic acids. Quantitative analysis highlighted that these were present at concentrations similar to, or exceeding, those of other plant materials noted as rich sources of phenolics such as Mulberry and Ginkgo leaves. Hop cone extracts contained lower levels of these compounds but had significantly higher levels of B-type procyanidins, catechins, prenylflavonoids and bitter resins.

Hop variety was shown to be a strong determinant of flavonol glycoside composition, with quercetin glycosides prevalent in Calypso but kaempferol glycosides predominant in Cascade and Contessa leaves. Even so, the distribution of glycosides was significantly different in each case such that phenolic concentration data clustered first by variety and then sub-clustered according to factors such as harvest year or stage of leaf development which had smaller and less consistent effects across the study. Although the number of young leaf samples (n=2) in the study was small their composition was substantially different to the mature leaves sampled at each stage of development and driven by their elevated concentrations of flavonol glycosides.

When compared to South African hop leaves analysed in **Chapter 4**, leaves from Yakima generally had higher flavonol glycoside levels but significantly lower total phenolic contents. It is unclear if this is attributed to variety or other factors but highlights the variability of hop

leaves as a source of natural phenolics, an important consideration for the potential commercial valorisation of hop leaf phenolics.

The higher phenolic contents of the South African hop leaves could be related to the hotter climate and the lack of irrigation (drought stress) compared to those grown in Yakima. Although this study found that correlations between climate variables and phenolic content were generally not significant and inconsistent between varieties, UV radiation and heat have been linked to the accumulation of different phenolic groups in leaves across a range of species such as lettuce (Sytar et al., 2018) and grape (Berli et al., 2010). The generally insignificant correlations reported in this study may be related to the confounding effect of leaf stage of development and climate variables on phenolic composition, which were difficult to separate based on the experimental design used.

Overall, this study highlights that hop leaves are a rich source of quercetin and kaempferol glycosides, as well as chlorogenic acids. Whilst flavonol glycoside composition was primarily driven by hop variety, chlorogenic acids showed less variety-specific variation.

Chapter 8 aimed to characterise the antioxidant activities of hop leaf and cone extracts and to identify the contributions of specific phenolic compounds to these activities. Hop cone extracts exhibited significantly higher antioxidant activities than all leaf extracts ($p>0.05$) which varied according to variety, stage of development and crop year. Correlation analysis between the antioxidant activities and phenolic composition of leaf extracts revealed catechins and B-type procyanidins had the strongest positive correlations ($p>0.05$) whilst kaempferol glycosides generally showed negative correlations. To investigate the causal relationship between compound concentration and antioxidant activities, phenolic standards were evaluated individually for antioxidant activities. Flavonol glycosides generally had significantly lower antioxidant activity compared to their respective aglycones ($P<0.05$), with

kaempferol 3-O-glycosides exhibiting negligible DPPH and FRAP activity. This was hypothesised to be related to the steric hinderance of glycosylation which may particularly affect the electron transfer-based antioxidant activity of kaempferol glycosides. Along with quercetin and kaempferol, B-type procyanidins had the highest activities of the pure compounds analysed, likely contributing to the significantly higher antioxidant activities of hop cone extracts compared to leaves.

Given that quercetin and kaempferol glycosides were the most abundant phenolics in hop leaf extracts (Chapter 6) and exhibited significantly lower antioxidant activities than their respective aglycones, it was aimed to evaluate hydrolytic treatment as a technique to enhance the antioxidant properties of hop leaf extracts. Snailase, an enzyme preparation comprised of cellulases, pectinases and proteases, was selected for glycoside hydrolysis trials as it has been shown to be more effective than comparable enzymes and acid hydrolysis (Wang et al., 2011). Three hop leaf samples (Cascade-Flower, S. Aroma-Harvest and S. Passion) with high but varied flavonol glycoside contents were purified using C18 SPE, treated with snailase, and analysed for differences in antioxidant activities and phenolic composition. Snailase treatment achieved high hydrolysis rates (>99%) and significant increases in antioxidant activity depending on glycoside composition and antioxidant mechanism. The Cascade leaf extract showed the greatest increases in antioxidant activities upon treatment, in particular for the FRAP assay (4.5*), likely due to its high kaempferol 3-O-rutinoside content (400.56 mg/g). These finding highlight the importance of hydrolytic treatment for hop leaf extracts for use as natural antioxidants depending on glycoside composition.

Overall, the work presented in the thesis has provided significant new information to support the future exploitation of hop co-products as sources of valuable phenolics with desirable antioxidant or other bioactive properties. Comprehensive identification and quantitative analysis of phenolics using LC-MS/MS enabled different sources of co-products across the

hop supply chain to be compared as source materials for phenolics valorisation. Extraction and purification strategies are presented which generated products which were demonstrably comparable to benchmark commercial phenolic extracts of plant materials in terms of their antioxidant activities and/or concentrations of key components of interest. The residue from commercial CO_2 extraction of hop pellets was shown to be most promising in terms of generating the most concentrated extracts. Hop leaf materials contained a distinct range of phenolic compounds relative to those present in the cones; the antioxidant activities of leaf extracts were found to vary according to source, variety, stage of development and treatment of leaf samples post-harvest. Antioxidant capacity of leaf extracts was significantly lower than for hop cone processing residues and thus a range of enzymatic treatments were investigated in attempts to enhance the extraction and antioxidant activity of leaf extracts. Snailase was identified as a promising enzyme treatment to cleave the predominant flavonol glycosides in leaf extracts and yield aglycones which have much greater antioxidant activities than the parent glycosides.

8.2-Future Work

Recommendations for future research:

- Proanthocyanidin depolymerisation

In this study PAD950 SPE generated hop phenolic extracts had higher proanthocyanidin contents, but lower antioxidant activities compared to commercially available extracts such as Grape OPC, Vitisol and Pineol. The observed differences between total proanthocyanidin content and the levels of B-type dimer and trimer procyanidins suggest that this fraction was predominantly comprised of high molecular weight proanthocyanidins, indicating the need to evaluate proanthocyanidin depolymerisation techniques. Effective green techniques such as catalytic hydrogenolysis which comply with food safety standards have been shown to be

effective and could be utilised to improve the antioxidant properties of hop proanthocyanidins.

- Evaluate of phenolics extracts for specific application

This study used in vitro antioxidant techniques to assess direct radical scavenging mechanisms relevant to various industries. However due to the complexities of oxidative reactions, and the diverse matrices of possible products, future research should evaluate extracts within specific product environments. This would provide a clearer understanding of efficacy across real-world applications and may highlight areas for optimisation. For example, phenolic extracts for beverage flavour stability are limited by their solubility in product. Many of the abundant phenolics quantified in co-product extracts have poor water solubility such as xanthohumol in CO_2R-HERK, and kaempferol, the main product of leaf glycoside hydrolysis. By evaluating these extracts in product it may indicate that encapsulation techniques are required to improve availability in the beverage, or that alternative applications are more promising.

- Economic feasibility of glycoside hydrolysis

This study highlighted that snailase glycoside hydrolysis was highly effective for improving the antioxidant capacity of leaf extracts. However, aglycone yields were lower than anticipated and the enzyme: substrate ratio evaluated is likely not economically feasible. Future work should investigate optimising incubation conditions to improve aglycone yields and demonstrate financial viability by evaluating lower enzyme: substrate ratios or by immobilization to improve reusability and stability of the enzyme.

- The impact of climate change on hop leaf phenolic content

Although correlations between climate variables and the phenolic content of hop leaves were generally insignificant in this study, it has been proposed that this may be due to limitations

in the experimental design. As climate change leads to elevated heat and UV radiation globally, future work should evaluate the effect of these changing climate conditions on the phenolic content of hop leaves in major growing regions. The accumulation of various phenolic compounds in response to elevated UV radiation, heat and drought stress suggests that the valorising hop leaves for their phenolic content could become more economically viable as the climate change progresses.

References:

ABASCAL, K., GANORA, L. & YARNELL, E. 2005. The effect of freeze‐drying and its implications for botanical medicine: a review. *Phytotherapy Research: An International Journal Devoted to Pharmacological and Toxicological Evaluation of Natural Product Derivatives,* 19**,** 655-660.

ABIKO, Y., PAUDEL, D. & UEHARA, O. 2022. Hops components and oral health. *Journal of Functional Foods,* 92**,** 105035.

ABRAM, V., ČEH, B., VIDMAR, M., HERCEZI, M., LAZIĆ, N., BUCIK, V., MOŽINA, S. S., KOŠIR, I. J., KAČ, M. & DEMŠAR, L. 2015. A comparison of antioxidant and antimicrobial activity between hop leaves and hop cones. *Industrial crops and products,* 64**,** 124-134.

ADEBOOYE, O. C., ALASHI, A. M. & ALUKO, R. E. 2018. A brief review on emerging trends in global polyphenol research. *Journal of Food Biochemistry,* 42**,** e12519.

AFONSO, S., PEREIRA, E. L., ARROBAS, M., RODRIGUES, M. Â. & CHOUPINA, A. 2023. Composts Obtained by Mixing Hop Leaves with Wheat Straw or Farmyard Manure Improved Soil Properties and Increased Microbial Communities. *Horticulturae,* 9**,** 1304.

AL-DHABI, N. A., PONMURUGAN, K. & JEGANATHAN, P. M. 2017. Development and validation of ultrasound-assisted solid-liquid extraction of phenolic compounds from waste spent coffee grounds. *Ultrasonics sonochemistry,* 34**,** 206-213.

ALFEO, V., SILEONI, V., BRAVI, E., BELARDI, I., DE FRANCESCO, G. & MARCONI, O. 2023. A sustainable valorisation of spent hops from dry-hopping. *LWT,* 186**,** 115248.

ALGAZZALI, V. & SHELLHAMMER, T. 2016. Bitterness intensity of oxidized hop acids: Humulinones and hulupones. *Journal of the American Society of Brewing Chemists,* 74**,** 36-43.

ALI, A., BASHMIL, Y. M., COTTRELL, J. J., SULERIA, H. A. & DUNSHEA, F. R. 2021. Lc-ms/ms-qtof screening and identification of phenolic compounds from australian grown herbs and their antioxidant potential. *Antioxidants,* 10**,** 1770.

ALMAGUER, C., SCHÖNBERGER, C., GASTL, M., ARENDT, E. K. & BECKER, T. 2014. Humulus lupulus–a story that begs to be told. A review. *Journal of the Institute of Brewing,* 120**,** 289-314.

ALONSO-ESTEBAN, J. I., PINELA, J., BARROS, L., ĆIRIĆ, A., SOKOVIĆ, M., CALHELHA, R. C., TORIJA-ISASA, E., DE CORTES SÁNCHEZ-MATA, M. & FERREIRA, I. C. 2019. Phenolic composition and antioxidant, antimicrobial and cytotoxic properties of hop (Humulus lupulus L.) Seeds. *Industrial Crops and Products,* 134**,** 154-159.

ALU'DATT, M. H., ALLI, I., EREIFEJ, K., ALHAMAD, M., AL-TAWAHA, A. R. & RABABAH, T. 2010. Optimisation, characterisation and quantification of phenolic compounds in olive cake. *Food Chemistry,* 123**,** 117-122.

ALVES, S. S. D., AFONSO, A. L. F., CARDOSO, S. M., SOUSA, M. J. & PEREIRA, O. R. 2021. Humulus lupulus L.: cosmetic application of extracts obtained from cones and vegetative parts. *1st Natural products application: Health, Cosmetic and Food***,** 57.

ANDERSEN, M. L. & SKIBSTED, L. H. 1998. Electron spin resonance spin trapping identification of radicals formed during aerobic forced aging of beer. *Journal of Agricultural and Food Chemistry,* 46**,** 1272-1275.

ANDERSEN, R. & SOWERS, J. 1968. Optimum conditions for bonding of plant phenols to insoluble polyvinylpyrrolidone. *Phytochemistry,* 7**,** 293-301.

ANDRADE, C., FERRERES, F., GOMES, N. G., GIL-IZQUIERDO, A., DUANGSRISAI, S., PEREIRA, D. M., ANDRADE, P. B. & VALENTÃO, P. 2022. Valorisation of the industrial waste of Chukrasia tabularis A. Juss.: Characterization of the leaves phenolic constituents and antidiabetic-like effects. *Industrial Crops and Products,* 185**,** 115100.

ANKE, J., PETEREIT, F., ENGELHARDT, C. & HENSEL, A. 2008. Procyanidins from Myrothamnus flabellifolia. *Natural Product Research,* 22**,** 1237-1248.

ARIMBOOR, R. & ARUMUGHAN, C. 2012. Effect of polymerization on antioxidant and xanthine oxidase inhibitory potential of sea buckthorn (H. rhamnoides) proanthocyanidins. *Journal of food science,* 77**,** C1036-C1041.

ARON, P. M. & SHELLHAMMER, T. H. 2010. A discussion of polyphenols in beer physical and flavour stability. *Journal of the Institute of Brewing,* 116**,** 369-380.

ASTRAY, G., GULLÓN, P., GULLÓN, B., MUNEKATA, P. E. & LORENZO, J. M. 2020. Humulus lupulus L. as a natural source of functional biomolecules. *Applied Sciences,* 10**,** 5074.

BAGCHI, D., BAGCHI, M., STOHS, S. J., DAS, D. K., RAY, S. D., KUSZYNSKI, C. A., JOSHI, S. S. & PRUESS, H. G. 2000. Free radicals and grape seed proanthocyanidin extract: importance in human health and disease prevention. *Toxicology,* 148**,** 187-197.

BAMFORTH, C. W. 2008. *Beer: Health and nutrition*, John Wiley & Sons.

BARTH‐HAAS‐GROUP 2023. The Barth Report.

BARTMAŃSKA, A., WAŁECKA-ZACHARSKA, E., TRONINA, T., POPŁOŃSKI, J., SORDON, S., BRZEZOWSKA, E., BANIA, J. & HUSZCZA, E. 2018. Antimicrobial properties of spent hops extracts, flavonoids isolated therefrom, and their derivatives. *Molecules,* 23**,** 2059.

BASTOLA, K. P., GURAGAIN, Y. N., BHADRIRAJU, V. & VADLANI, P. V. 2017. Evaluation of standards and interfering compounds in the determination of phenolics by Folin-Ciocalteu assay method for effective bioprocessing of biomass. *American Journal of Analytical Chemistry,* 8**,** 416-431.

BEDINI, S., FLAMINI, G., GIRARDI, J., COSCI, F. & CONTI, B. 2015. Not just for beer: Evaluation of spent hops (Humulus lupulus L.) as a source of eco-friendly repellents for insect pests of stored foods. *Journal of pest science,* 88**,** 583-592.

BELITZ, H.-D., GROSCH, W. & SCHIEBERLE, P. 2008. *Food chemistry*, Springer Science & Business Media.

BENZIE, I. F. & STRAIN, J. J. 1996. The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": the FRAP assay. *Analytical biochemistry,* 239**,** 70-76.

BERENGUER, C. V., ANDRADE, C., PEREIRA, J. A., PERESTRELO, R. & CÂMARA, J. S. 2022. Current challenges in the sustainable valorisation of agri-food wastes: a review. *Processes,* 11**,** 20.

BERLI, F. J., MORENO, D., PICCOLI, P., HESPANHOL‐VIANA, L., SILVA, M. F., BRESSAN‐SMITH, R., CAVAGNARO, J. B. & BOTTINI, R. 2010. Abscisic acid is involved in the response of grape (Vitis vinifera L.) cv. Malbec leaf tissues to ultraviolet‐B radiation by enhancing ultraviolet‐absorbing compounds, antioxidant enzymes and membrane sterols. *Plant, Cell & Environment,* 33**,** 1-10.

BEZERRA, R. M. & DIAS, A. A. 2005. Enzymatic kinetic of cellulose hydrolysis: inhibition by ethanol and cellobiose. *Applied biochemistry and biotechnology,* 126**,** 49-59.

BIENDL, M. Isolation of prenylflavonoids from hops. III International Humulus Symposium 1010, 2012. 131-140.

BIENDL, M., ENGELHARD, B., FORSTER, A., GAHR, A., LUTZ, A., MITTER, W., SCHMIDT, R. & SCHÖNBERGER, C. 2015. *Hops: their cultivation, composition and usage*, Fachverlag Hans Carl.

BOHR, G., GERHÄUSER, C., KNAUFT, J., ZAPP, J. & BECKER, H. 2005. Anti-inflammatory Acylphloroglucinol Derivatives from Hops (Humulus l upulus). *Journal of natural products,* 68**,** 1545-1548.

BONCLER, M., RÓŻALSKI, M., KRAJEWSKA, U., PODSĘDEK, A. & WATALA, C. 2014. Comparison of PrestoBlue and MTT assays of cellular viability in the assessment of anti-proliferative effects of plant extracts on human endothelial cells. *Journal of pharmacological and toxicological methods,* 69**,** 9-16.

BORS, W., MICHEL, C. & STETTMAIER, K. 2000. Electron paramagnetic resonance studies of radical species of proanthocyanidins and gallate esters. *Archives of Biochemistry and Biophysics,* 374**,** 347-355.
BRACA, A., FICO, G., MORELLI, I., DE SIMONE, F., TOMÈ, F. & DE TOMMASI, N. 2003. Antioxidant and free radical scavenging activity of flavonol glycosides from different Aconitum species. *Journal of Ethnopharmacology,* 86**,** 63-67.
BRAMATI, L., AQUILANO, F. & PIETTA, P. 2003. Unfermented rooibos tea: quantitative characterization of flavonoids by HPLC− UV and determination of the total antioxidant activity. *Journal of agricultural and food chemistry,* 51**,** 7472-7474.
BRANOWSKI, K. & ROLNO-SPOZYWCZEGO, I. B. P. 2007. Polyphenols with antioxidant and positive to health effects in some hop varieties and hop granulates and extracts used for production of Polish beer. *Prace Instytutow i Laboratoriow Badawczych Przemyslu Spozywczego (Poland)*.
BRAVI, E., FRANCESCO, G. D., SILEONI, V., PERRETTI, G., GALGANO, F. & MARCONI, O. 2021. Brewing by-product upcycling potential: Nutritionally valuable compounds and antioxidant activity evaluation. *Antioxidants,* 10**,** 165.
BRISTOL, C. 2022. A Cradle-to-Gate Life Cycle Assessment of American Hops in the Craft Beer Supply Chain.
BURTON, J., STEVENS, R. & ELVIDGE, J. 1964. 185. Chemistry of hop constituents. Part XVIII. Hulupinic acid. *Journal of the Chemical Society (Resumed)***,** 952-955.
CABAN, M., CHOJNACKA, K., OWCZAREK, K., LASKOWSKA, J., FICHNA, J., PODSĘDEK, A., SOSNOWSKA, D. & LEWANDOWSKA, U. 2020. Spent hops (Humulus lupulus L.) extract as modulator of the inflammatory response in lipopolysaccharide stimulated raw 264.7 macrophages. *Journal of Physiology and Pharmacology,* 70.
CAMPONE, L., CELANO, R., RIZZO, S., PICCINELLI, A. L., RASTRELLI, L. & RUSSO, M. 2020. Development of an enriched polyphenol (natural antioxidant) extract from orange juice (Citrus sinensis) by adsorption on macroporous resins. *Journal of Food Quality,* 2020**,** 1251957.
CANGELONI, L., BONECHI, C., LEONE, G., CONSUMI, M., ANDREASSI, M., MAGNANI, A., ROSSI, C. & TAMASI, G. 2022. Characterization of extracts of coffee leaves (Coffea arabica L.) by spectroscopic and chromatographic/spectrometric techniques. *Foods,* 11**,** 2495.
CASAZZA, A. A., ALIAKBARIAN, B. & PEREGO, P. 2011. Recovery of phenolic compounds from grape seeds: Effect of extraction time and solid–liquid ratio. *Natural Product Research,* 25**,** 1751-1761.
CASSANO, A., CONIDI, C., RUBY-FIGUEROA, R. & CASTRO-MUÑOZ, R. 2018. Nanofiltration and tight ultrafiltration membranes for the recovery of polyphenols from agro-food by-products. *International Journal of Molecular Sciences,* 19**,** 351.
CEH, B., KAC, M., KOŠIR, I. J. & ABRAM, V. 2007. Relationships between xanthohumol and polyphenol content in hop leaves and hop cones with regard to water supply and cultivar. *International Journal of Molecular Sciences,* 8**,** 989-1000.
CENSI, R., VARGAS PEREGRINA, D., GIGLIOBIANCO, M. R., LUPIDI, G., ANGELONI, C., PRUCCOLI, L., TAROZZI, A. & DI MARTINO, P. 2021. New Antioxidant Ingredients from Brewery By-Products for Cosmetic Formulations. *Cosmetics,* 8**,** 96.
CHADWICK, L. R., NIKOLIC, D., BURDETTE, J. E., OVERK, C. R., BOLTON, J. L., VAN BREEMEN, R. B., FRÖHLICH, R., FONG, H. H., FARNSWORTH, N. R. & PAULI, G. F. 2004. Estrogens and Congeners from Spent Hops (Humulus l upulus). *Journal of natural products,* 67**,** 2024-2032.
CHEN, W., LIU, G. & ZHANG, Y. 2024. Production of Rhamnosyl Icariside II by snailase hydrolysis of Epimedium wushanense extracts. *Heliyon,* 10.

CHENOT, C., SIMON, M., DUSART, A. & COLLIN, S. 2023. Exploring Hop Varieties with Discriminating Flavan-3-ol Profiles Likely to Improve Color and Colloidal Stability of Beers. *Beverages,* 9**,** 67.

CHIANCONE, B., GUARRASI, V., LETO, L., DEL VECCHIO, L., CALANI, L., GANINO, T., GALAVERNI, M. & CIRLINI, M. 2023. Vitro-derived hop (Humulus lupulus L.) leaves and roots as source of bioactive compounds: antioxidant activity and polyphenolic profile. *Plant Cell, Tissue and Organ Culture (PCTOC),* 153**,** 295-306.

CHIRINOS, R., ROGEZ, H., CAMPOS, D., PEDRESCHI, R. & LARONDELLE, Y. 2007. Optimization of extraction conditions of antioxidant phenolic compounds from mashua (Tropaeolum tuberosum Ruíz & Pavón) tubers. *Separation and Purification Technology,* 55**,** 217-225.

CHLOPICKA, J., PASKO, P., GORINSTEIN, S., JEDRYAS, A. & ZAGRODZKI, P. 2012. Total phenolic and total flavonoid content, antioxidant activity and sensory evaluation of pseudocereal breads. *LWT-Food Science and Technology,* 46**,** 548-555.

CHOI, J. Y., DESTA, K. T., LEE, S. J., KIM, Y. H., SHIN, S. C., KIM, G. S., LEE, S. J., SHIM, J. H., HACIMÜFTÜOĞLU, A. & ABD EL‐ATY, A. 2018. LC‐MS/MS profiling of polyphenol‐enriched leaf, stem and root extracts of Korean Humulus japonicus Siebold & Zucc and determination of their antioxidant effects. *Biomedical Chromatography,* 32**,** e4171.

CIFUENTES-CABEZAS, M., SANCHEZ-ARÉVALO, C. M., MENDOZA-ROCA, J. A., VINCENT-VELA, M. C. & ÁLVAREZ-BLANCO, S. 2022. Recovery of phenolic compounds from olive oil washing wastewater by adsorption/desorption process. *Separation and Purification Technology,* 298**,** 121562.

CLIFFORD, M. N. 1999. Chlorogenic acids and other cinnamates–nature, occurrence and dietary burden. *Journal of the Science of Food and Agriculture,* 79**,** 362-372.

CONIDI, C., CASSANO, A., CAIAZZO, F. & DRIOLI, E. 2017. Separation and purification of phenolic compounds from pomegranate juice by ultrafiltration and nanofiltration membranes. *Journal of Food Engineering,* 195**,** 1-13.

CONIDI, C., DRIOLI, E. & CASSANO, A. 2018. Membrane-based agro-food production processes for polyphenol separation, purification and concentration. *Current Opinion in Food Science,* 23**,** 149-164.

CORTESE, M., GIGLIOBIANCO, M. R., PEREGRINA, D. V., SAGRATINI, G., CENSI, R. & DI MARTINO, P. 2020. Quantification of phenolic compounds in different types of crafts beers, worts, starting and spent ingredients by liquid chromatography-tandem mass spectrometry. *Journal of Chromatography A,* 1612**,** 460622.

CUI, P., DOU, T.-Y., SUN, Y.-P., LI, S.-Y., FENG, L., ZOU, L.-W., WANG, P., HAO, D.-C., GE, G.-B. & YANG, L. 2016. Efficient enzymatic preparation of esculentoside B following condition optimization by response surface methodology. *Journal of Molecular Catalysis B: Enzymatic,* 130**,** 25-31.

DA SILVA, R. G., ALMEIDA, T. C., REIS, A. C. C., FILHO, S. A. V., BRANDÃO, G. C., DA SILVA, G. N., DE SOUSA, H. C., DE ALMEIDA, V. L., LOPES, J. C. D. & DE SOUZA, G. H. B. 2021. In silico pharmacological prediction and cytotoxicity of flavonoids glycosides identified by UPLC-DAD-ESI-MS/MS in extracts of Humulus lupulus leaves cultivated in Brazil. *Natural Product Research,* 35**,** 5918-5923.

DADIC, M. & LAVALLEE, J. 1983. The use of Polyclar AT (PVPP) in brewing. *Journal of the American Society of Brewing Chemists,* 41**,** 141-147.

DAVID, I. G., BIZGAN, A.-M. C., POPA, D. E., BULEANDRA, M., MOLDOVAN, Z., BADEA, I. A., TEKINER, T. A., BASAGA, H. & CIUCU, A. A. 2015. Rapid determination of total polyphenolic content in tea samples based on caffeic acid voltammetric behaviour on a disposable graphite electrode. *Food Chemistry,* 173**,** 1059-1065.

DE ANDRADE SILVA, G. V., AREND, G. D., ZIELINSKI, A. A. F., DI LUCCIO, M. & AMBROSI, A. 2023. Xanthohumol properties and strategies for extraction from hops and brewery residues: A review. *Food Chemistry,* 404**,** 134629.

DE ARAÚJO, M. E. M. B., FRANCO, Y. E. M., ALBERTO, T. G., SOBREIRO, M. A., CONRADO, M. A., PRIOLLI, D. G., SAWAYA, A. C. F., RUIZ, A. L. T., DE CARVALHO, J. E. & DE OLIVEIRA CARVALHO, P. 2013. Enzymatic de-glycosylation of rutin improves its antioxidant and antiproliferative activities. *Food chemistry,* 141**,** 266-273.

DE CAMARGO, A. C., REGITANO-D'ARCE, M. A. B., BIASOTO, A. C. T. & SHAHIDI, F. 2016. Enzyme-assisted extraction of phenolics from winemaking by-products: Antioxidant potential and inhibition of alpha-glucosidase and lipase activities. *Food chemistry,* 212**,** 395-402.

DE KEUKELEIRE, J., OOMS, G., HEYERICK, A., ROLDAN-RUIZ, I., VAN BOCKSTAELE, E. & DE KEUKELEIRE, D. 2003. Formation and accumulation of α-acids, β-acids, desmethylxanthohumol, and xanthohumol during flowering of hops (Humulus lupulus L.). *Journal of Agricultural and Food Chemistry,* 51**,** 4436-4441.

DESHPANDE, S. & CHERYAN, M. 1985. Evaluation of vanillin assay for tannin analysis of dry beans. *Journal of Food Science,* 50**,** 905-910.

DÍAZ, N., AQUEVEQUE, P. M., VALLEJOS-ALMIRALL, A., RADRIGÁN, R., ZÚÑIGA-LÓPEZ, M. C. & FOLCH-CANO, C. 2022. Antioxidant Compound Adsorption in Polyvinylpolypyrrolidone from Chilean Carménère, Cabernet Sauvignon, and Merlot Grape Pomaces as Potential By-Products. *Antioxidants,* 11**,** 2017.

DJOUMBOU FEUNANG, Y., EISNER, R., KNOX, C., CHEPELEV, L., HASTINGS, J., OWEN, G., FAHY, E., STEINBECK, C., SUBRAMANIAN, S. & BOLTON, E. 2016. ClassyFire: automated chemical classification with a comprehensive, computable taxonomy. *Journal of cheminformatics,* 8**,** 1-20.

DOMÍNGUEZ-RODRÍGUEZ, G., MARINA, M. L. & PLAZA, M. 2021. Enzyme-assisted extraction of bioactive non-extractable polyphenols from sweet cherry (Prunus avium L.) pomace. *Food chemistry,* 339**,** 128086.

DONG, Z.-B., LIANG, Y.-R., FAN, F.-Y., YE, J.-H., ZHENG, X.-Q. & LU, J.-L. 2011. Adsorption behavior of the catechins and caffeine onto polyvinylpolypyrrolidone. *Journal of agricultural and food chemistry,* 59**,** 4238-4247.

DOS SANTOS SCHOLZ, M. B., KITZBERGER, C. S. G., DURAND, N. & RAKOCEVIC, M. 2018. From the field to coffee cup: Impact of planting design on chlorogenic acid isomers and other compounds in coffee beans and sensory attributes of coffee beverage. *European Food Research and Technology,* 244**,** 1793-1802.

DOSTÁLEK, P., KARABÍN, M. & JELÍNEK, L. 2017. Hop phytochemicals and their potential role in metabolic syndrome prevention and therapy. *Molecules,* 22**,** 1761.

DROSOU, C., KYRIAKOPOULOU, K., BIMPILAS, A., TSIMOGIANNIS, D. & KROKIDA, M. 2015. A comparative study on different extraction techniques to recover red grape pomace polyphenols from vinification byproducts. *Industrial Crops and Products,* 75**,** 141-149.

DUDONNE, S., VITRAC, X., COUTIERE, P., WOILLEZ, M. & MÉRILLON, J.-M. 2009. Comparative study of antioxidant properties and total phenolic content of 30 plant extracts of industrial interest using DPPH, ABTS, FRAP, SOD, and ORAC assays. *Journal of agricultural and food chemistry,* 57**,** 1768-1774.

DÜHRKOP, K., FLEISCHAUER, M., LUDWIG, M., AKSENOV, A. A., MELNIK, A. V., MEUSEL, M., DORRESTEIN, P. C., ROUSU, J. & BÖCKER, S. 2019. SIRIUS 4: a rapid tool for turning tandem mass spectra into metabolite structure information. *Nature methods,* 16**,** 299-302.

DÜHRKOP, K., NOTHIAS, L.-F., FLEISCHAUER, M., REHER, R., LUDWIG, M., HOFFMANN, M. A., PETRAS, D., GERWICK, W. H., ROUSU, J. & DORRESTEIN, P. C. 2021. Systematic

classification of unknown metabolites using high-resolution fragmentation mass spectra. *Nature biotechnology,* 39**,** 462-471.
DÜHRKOP, K., SHEN, H., MEUSEL, M., ROUSU, J. & BÖCKER, S. 2015. Searching molecular structure databases with tandem mass spectra using CSI: FingerID. *Proceedings of the National Academy of Sciences,* 112**,** 12580-12585.
DUŠEK, M., JANDOVSKÁ, V., MIKYŠKA, A. & OLŠOVSKÁ, J. 2021. Novel and efficient approach to identify hop cultivars (Humulus lupulus L.) using cultivar identification diagram strategy based on fingerprint of flavonol di-and tri-O-glycosides. *European Food Research and Technology,* 247**,** 651-662.
EBC. 2018. *7.15-Xanthohumol in Hops and Hop Products by HPLC* [Online]. [Accessed 01/01/2025].
FERNANDEZ-OCHOA, A., DE LA LUZ CÁDIZ-GURREA, M. & SEGURA CARRETERO, A. 2022. Comprehensive identification of plant polyphenols by LC-MS. *Mass Spectrometry for Food Analysis.* Springer.
FERRANDO, M., RÓZEK, A., ACHAERANDIO, I. & GÜELL, C. 2011. Grape phenolic infusion into solid foods: studies on mass transfer and antioxidant capacity. *Procedia Food Science,* 1**,** 1494-1501.
FERREIRA, S. S., ALVES, A. J., FILIPE-RIBEIRO, L., COSME, F. & NUNES, F. M. 2018. Holistic and sustainable approach for recycling and valorization of polyvinylpolypyrrolidone used in wine fining. *ACS Sustainable Chemistry & Engineering,* 6**,** 14599-14606.
FLOEGEL, A., KIM, D.-O., CHUNG, S.-J., KOO, S. I. & CHUN, O. K. 2011. Comparison of ABTS/DPPH assays to measure antioxidant capacity in popular antioxidant-rich US foods. *Journal of food composition and analysis,* 24**,** 1043-1048.
FLÓREZ‐FERNÁNDEZ, N., CASAS, M. P., JESÚS GONZÁLEZ‐MUÑOZ, M. & DOMÍNGUEZ, H. 2019. Microwave hydrogravity pretreatment of Sargassum muticum before solvent extraction of antioxidant and antiobesity compounds. *Journal of Chemical Technology & Biotechnology,* 94**,** 256-264.
FOLCH-CANO, C., OLEA-AZAR, C. & SPEISKY, H. 2013. Structural and thermodynamic factors on the adsorption process of phenolic compounds onto polyvinylpolypyrrolidone. *Colloids and Surfaces A: Physicochemical and Engineering Aspects,* 418**,** 105-111.
FORINO, M., PACE, S., CHIANESE, G., SANTAGOSTINI, L., WERNER, M., WEINIGEL, C., RUMMLER, S., FICO, G., WERZ, O. & TAGLIALATELA-SCAFATI, O. 2016. Humudifucol and bioactive prenylated polyphenols from hops (Humulus lupulus cv."Cascade"). *Journal of natural products,* 79**,** 590-597.
FORSTER, A., BECK, B. & SCHMIDT, R. Investigations on hop polyphenols. Proc. Eur. Brew. Conv. Congr., Brussels, 1995. IRL Press Oxford, 143-150.
FORSTER, A., BECK, B., SCHMIDT, R., JANSEN, C. & MELLENTHIN, A. 2002. On the composition of low molecular polyphenols in different varieties of hops and from two growing areas.
FORSTER, A. & GAHR, A. 2019. Comparison of hop varieties: Herkules and Polaris. *Brauwelt International,* 37**,** 182-185.
FRAGA, C. G., CROFT, K. D., KENNEDY, D. O. & TOMÁS-BARBERÁN, F. A. 2019. The effects of polyphenols and other bioactives on human health. *Food & function,* 10**,** 514-528.
FRANKEL, E. N. & MEYER, A. S. 2000. The problems of using one‐dimensional methods to evaluate multifunctional food and biological antioxidants. *Journal of the Science of Food and Agriculture,* 80**,** 1925-1941.
FRASCARI, D., BACCA, A. E. M., ZAMA, F., BERTIN, L., FAVA, F. & PINELLI, D. 2016. Olive mill wastewater valorisation through phenolic compounds adsorption in a continuous flow column. *Chemical Engineering Journal,* 283**,** 293-303.
FREEMAN, G., GOURRIEREC, X. L., PATEL, A., DAWSON, M., POWELL‐EVANS, M., SKIPPER, A., EVANS, C., BOULTON, C., GRIMMETT, C. & BARON, J. 2003. Improving the

effectiveness of isinglass finings for beer clarification by optimisation of the mixing process. Part 1: Laboratory scale experiments. *Journal of the Institute of Brewing,* 109**,** 309-317.

FROSI, I., MONTAGNA, I., COLOMBO, R., MILANESE, C. & PAPETTI, A. 2021. Recovery of chlorogenic acids from agri-food wastes: Updates on green extraction techniques. *Molecules,* 26**,** 4515.

GADON, A., LINFORTH, R., HARDING, S. E. & COOK, D. 2019a. Characterisation of high molecular weight hop proanthocyanidins using Analytical Ultracentrifugation. *Scientific Reports,* 9**,** 12650.

GADON, A., LINFORTH, R., HARDING, S. E. & COOK, D. 2019b. Characterisation of high molecular weight hop proanthocyanidins using Analytical Ultracentrifugation. *Scientific reports,* 9**,** 1-8.

GARRIDO, J. & BORGES, F. 2013. Wine and grape polyphenols—A chemical perspective. *Food research international,* 54**,** 1844-1858.

GE, X., WU, Z., MANZOLI, M., WU, Z. & CRAVOTTO, G. 2020. Feasibility and the mechanism of desorption of phenolic compounds from activated carbons. *Industrial & Engineering Chemistry Research,* 59**,** 12223-12231.

GERHÄUSER, C., ALT, A., KLIMO, K., KNAUFT, J., FRANK, N. & BECKER, H. 2002. Isolation and potential cancer chemopreventive activities of phenolic compounds of beer. *Phytochemistry Reviews,* 1**,** 369-377.

GESSNER, M. O. & STEINER, D. 2005. Acid butanol assay for proanthocyanidins (condensed tannins). *Methods to study litter decomposition: a practical guide***,** 107-114.

GIRISA, S., SAIKIA, Q., BORDOLOI, D., BANIK, K., MONISHA, J., DAIMARY, U. D., VERMA, E., AHN, K. S. & KUNNUMAKKARA, A. B. 2021. Xanthohumol from Hop: Hope for cancer prevention and treatment. *IUBMB life,* 73**,** 1016-1044.

GLIGOR, O., MOCAN, A., MOLDOVAN, C., LOCATELLI, M., CRIȘAN, G. & FERREIRA, I. C. 2019. Enzyme-assisted extractions of polyphenols–A comprehensive review. *Trends in Food Science & Technology,* 88**,** 302-315.

GOIRIS, K., JASKULA-GOIRIS, B., SYRYN, E., VAN OPSTAELE, F., DE ROUCK, G., AERTS, G. & DE COOMAN, L. 2014. The flavoring potential of hop polyphenols in beer. *Journal of the American Society of Brewing Chemists,* 72**,** 135-142.

GORJANOVIĆ, S., PASTOR, F. T., VASIC, R., NOVAKOVIĆ, M., SIMONOVIĆ, M., MILIĆ, S. & SUZNJEVIC, D. 2013. Electrochemical versus spectrophotometric assessment of antioxidant activity of hop (Humulus lupulus L.) products and individual compounds. *Journal of Agricultural and Food Chemistry,* 61**,** 9089-9096.

GRANATO, D., SANTOS, J. S., ESCHER, G. B., FERREIRA, B. L. & MAGGIO, R. M. 2018. Use of principal component analysis (PCA) and hierarchical cluster analysis (HCA) for multivariate association between bioactive compounds and functional properties in foods: A critical perspective. *Trends in Food Science & Technology,* 72**,** 83-90.

GRAY, D., UPTON, R., CHANDRA, A., PORTER, A. & HARRIS, R. 2006. Quantitative analysis of flavonol glycosides in Ginkgo biloba: a comparison of two analytical methods. *Phytochemical Analysis: An International Journal of Plant Chemical and Biochemical Techniques,* 17**,** 56-62.

GRUDNIEWSKA, A. & PASTYRCZYK, N. 2023. New insight for spent hops utilization: Simultaneous extraction of protein and xanthohumol using deep eutectic solvents. *Biomass Conversion and Biorefinery,* 13**,** 14975-14986.

GÜLZ, P.-G., MÜLLER, E., HERRMANN, T. & LÖSEL, P. 1993. Epicuticular leaf waxes of the hop (Humulus lupulus). Chemical composition and surface structures. *Zeitschrift für Naturforschung C,* 48**,** 689-696.

HAM, E. E. 2023. Alternative plant and fungi sourced fining agents in beer: A dissertation submitted in partial fulfilment of the requirements for the Degree of Master at Lincoln University.

HARADA, K., MAEDA, T., HASEGAWA, Y., TOKUNAGA, T., OGAWA, S., FUKUDA, K., NAGATSUKA, N., NAGAO, K. & UENO, S. 2011. Antioxidant activity of the giant jellyfish Nemopilema nomurai measured by the oxygen radical absorbance capacity and hydroxyl radical averting capacity methods. *Molecular Medicine Reports,* 4**,** 919-922.

HEIM, K. E., TAGLIAFERRO, A. R. & BOBILYA, D. J. 2002. Flavonoid antioxidants: chemistry, metabolism and structure-activity relationships. *The Journal of nutritional biochemistry,* 13**,** 572-584.

HELMJA, K., VAHER, M., PÜSSA, T., KAMSOL, K., ORAV, A. & KALJURAND, M. 2007. Bioactive components of the hop strobilus: Comparison of different extraction methods by capillary electrophoretic and chromatographic methods. *Journal of Chromatography A,* 1155**,** 222-229.

HENRION, M., SERVAES, M., THIELECKE, F. & FOGLIANO, V. 2018. Application of the QUENCHER methodology to the food industry. *Food Chemistry,* 240**,** 951-958.

HIDALGO, A. M., MACARIO, J. A., ABELLÁN-BAEZA, M., SÁNCHEZ-MOYA, T., LÓPEZ-NICOLÁS, R. & MARÍN-INIESTA, F. 2024. Pomegranate Juice Clarification Using Ultrafiltration: Influence of the Type of Variety and Degree of Ripeness. *Separations,* 11**,** 134.

HOLLMAN, P. C., BIJSMAN, M. N., VAN GAMEREN, Y., CNOSSEN, E. P., DE VRIES, J. H. & KATAN, M. B. 1999. The sugar moiety is a major determinant of the absorption of dietary flavonoid glycosides in man. *Free radical research,* 31**,** 569-573.

HOPSTEINER. *Variety data sheets* [Online]. Available: https://www.hopsteiner.com/variety-data-sheets/ [Accessed 28/10/24 2024].

HU, S., YIN, J., NIE, S., WANG, J., PHILLIPS, G. O., XIE, M. & CUI, S. W. 2016. In vitro evaluation of the antioxidant activities of carbohydrates. *Bioactive carbohydrates and dietary fibre,* 7**,** 19-27.

HU, Y., LIU, X., SONG, Y., ZHANG, Y., LI, W., ZHANG, L., WANG, A., SU, Q., YANG, Z. & ZOU, L. 2024. Exploring the anti‐inflammatory ingredients and potential of golden buckwheat (Fagopyrum dibotrys) on the TLR4/NLRP3 pathway in acute lung injury. *Food Science & Nutrition*.

HUAMÁN-CASTILLA, N. L., MARIOTTI-CELIS, M. S., MARTÍNEZ-CIFUENTES, M. & PÉREZ-CORREA, J. R. 2020. Glycerol as alternative co-solvent for water extraction of polyphenols from Carménère pomace: Hot pressurized liquid extraction and computational chemistry calculations. *Biomolecules,* 10**,** 474.

HUANG, D., OU, B., HAMPSCH-WOODILL, M., FLANAGAN, J. A. & DEEMER, E. K. 2002a. Development and validation of oxygen radical absorbance capacity assay for lipophilic antioxidants using randomly methylated β-cyclodextrin as the solubility enhancer. *Journal of agricultural and Food Chemistry,* 50**,** 1815-1821.

HUANG, D., OU, B., HAMPSCH-WOODILL, M., FLANAGAN, J. A. & PRIOR, R. L. 2002b. High-throughput assay of oxygen radical absorbance capacity (ORAC) using a multichannel liquid handling system coupled with a microplate fluorescence reader in 96-well format. *Journal of agricultural and food chemistry,* 50**,** 4437-4444.

HUGHES, P. & SIMPSON, W. 1993. Production and composition of hop products. *Technical quarterly-master brewers association of the Americas,* 30**,** 146-146.

HUYNH, N. T., SMAGGHE, G., GONZALES, G. B., VAN CAMP, J. & RAES, K. 2014. Enzyme-assisted extraction enhancing the phenolic release from cauliflower (Brassica oleracea L. var. botrytis) outer leaves. *Journal of Agricultural and Food Chemistry,* 62**,** 7468-7476.

IGLESIAS, A., MARTINEZ, P. G., RAMIREZ, C., MITTON, G., ARCERITO, F. M., FANGIO, M., CHURIO, M., FUSELLI, S., FANOVICH, A. & EGUARAS, M. 2021. Valorization of hop leaves for development of eco-friendly bee pesticides. *Apidologie,* 52**,** 186-198.

INGLETT, G. E., ROSE, D. J., CHEN, D., STEVENSON, D. G. & BISWAS, A. 2010. Phenolic content and antioxidant activity of extracts from whole buckwheat (Fagopyrum

esculentum Möench) with or without microwave irradiation. *Food Chemistry,* 119**,** 1216-1219.
INUI, T., OKUMURA, K., MATSUI, H., HOSOYA, T. & KUMAZAWA, S. 2017. Effect of harvest time on some in vitro functional properties of hop polyphenols. *Food chemistry,* 225**,** 69-76.
ISLAM, M. R., KAMAL, M. M., KABIR, M. R., HASAN, M. M., HAQUE, A. R. & HASAN, S. K. 2023. Phenolic compounds and antioxidants activity of banana peel extracts: Testing and optimization of enzyme-assisted conditions. *Measurement: Food,* 10**,** 100085.
JACQUIN, J., MOUREU, S., DEWEER, C., HAKEM, A., PAGUET, A.-S., BONNEAU, N., BORDAGE, S., DERMONT, C., SAHPAZ, S. & MUCHEMBLED, J. 2022. Hop (Humulus lupulus L.) Specialized Metabolites: Extraction, Purification, Characterization in Different Plant Parts and In Vitro Evaluation of Anti-Oomycete Activities against Phytophthora infestans. *Agronomy,* 12**,** 2826.
JANKOWIAK, L., VAN AVERMAETE, I., BOOM, R. & VAN DER GOOT, A. J. 2015. Adsorption of isoflavones onto PVPP in the presence of a soy matrix. *Separation and Purification Technology,* 149**,** 479-487.
JASKULA-GOIRIS, B., GOIRIS, K., SYRYN, E., VAN OPSTAELE, F., DE ROUCK, G., AERTS, G. & DE COOMAN, L. 2014. The use of hop polyphenols during brewing to improve flavor quality and stability of pilsner beer. *Journal of the American Society of Brewing Chemists,* 72**,** 175-183.
JELÍNEK, L., KARABÍN, M., KOTLÍKOVÁ, B., HUDCOVÁ, T. & DOSTÁLEK, P. 2014. Application of a hop by‐product in brewing: reduction in the level of haze‐active prolamines and improved antioxidant properties of the beer. *Journal of the Institute of Brewing,* 120**,** 99-104.
JELINEK, L., ŠNEBERGER, M., KARABIN, M. & DOSTALEK, P. 2010. Comparison of Czech hop cultivars based on their contenst of secondary metabolites. *Czech Journal of Food Sciences,* 28**,** 309-316.
JERKOVIC, V., CALLEMIEN, D. & COLLIN, S. 2005. Determination of stilbenes in hop pellets from different cultivars. *Journal of agricultural and food chemistry,* 53**,** 4202-4206.
JIA, D., TIAN, X., CHEN, Y., LIU, J., WANG, M., HAO, Z., WANG, C. & ZHAO, D. 2024. Preparation of enzymatic hydrolysates of mulberry leaf flavonoids and investigation into its treatment and mechanism for zebrafish inflammatory bowel disease. *Fish & Shellfish Immunology***,** 109960.
JIANG, H., LI, J., CHEN, L. & WANG, Z. 2020. Adsorption and desorption of chlorogenic acid by macroporous adsorbent resins during extraction of Eucommia ulmoides leaves. *Industrial crops and products,* 149**,** 112336.
JU, W.-T., KWON, O.-C., KIM, H.-B., SUNG, G.-B., KIM, H.-W. & KIM, Y.-S. 2018. Qualitative and quantitative analysis of flavonoids from 12 species of Korean mulberry leaves. *Journal of food science and Technology,* 55**,** 1789-1796.
KALAM, S., ABU-KHAMSIN, S. A., KAMAL, M. S. & PATIL, S. 2021. Surfactant adsorption isotherms: A review. *ACS omega,* 6**,** 32342-32348.
KAMMERER, J., KAMMERER, D. R., JENSEN, U. & CARLE, R. 2010. Interaction of apple polyphenols in a multi-compound system upon adsorption onto a food-grade resin. *Journal of food engineering,* 96**,** 544-554.
KARABÍN, M., HAIMANNOVÁ, T., FIALOVÁ, K., JELÍNEK, L. & DOSTÁLEK, P. 2021. Preparation of Hop Estrogen-Active Material for Production of Food Supplements. *Molecules,* 26**,** 6065.
KARABÍN, M., HANKO, V., NEŠPOR, J., JELÍNEK, L. & DOSTÁLEK, P. 2018. Hop tannin extract: a promising tool for acceleration of lautering. *Journal of the Institute of Brewing,* 124**,** 374-380.

KARABIN, M., HUDCOVA, T., JELINEK, L. & DOSTALEK, P. 2015. Biotransformations and biological activities of hop flavonoids. *Biotechnology advances,* 33**,** 1063-1090.
KATSUBE, T., TSURUNAGA, Y., SUGIYAMA, M., FURUNO, T. & YAMASAKI, Y. 2009. Effect of air-drying temperature on antioxidant capacity and stability of polyphenolic compounds in mulberry (Morus alba L.) leaves. *Food Chemistry,* 113**,** 964-969.
KAVALIER, A. R., LITT, A., MA, C., PITRA, N. J., COLES, M. C., KENNELLY, E. J. & MATTHEWS, P. D. 2011. Phytochemical and morphological characterization of hop (Humulus lupulus L.) cones over five developmental stages using high performance liquid chromatography coupled to time-of-flight mass spectrometry, ultrahigh performance liquid chromatography photodiode array detection, and light microscopy techniques. *Journal of agricultural and food chemistry,* 59**,** 4783-4793.
KELLY, N., KELLY, A. & O'MAHONY, J. 2019. Strategies for enrichment and purification of polyphenols from fruit-based materials. *Trends in Food Science & Technology,* 83**,** 248-258.
KELM, M. A., HAMMERSTONE, J. F. & SCHMITZ, H. H. 2005. Identification and quantitation of flavanols and proanthocyanidins in foods: How good are the datas? *Clinical and Developmental Immunology,* 12**,** 35-41.
KENNEDY, S. H., MANEVICH, Y. & BIAGLOW, J. 1995. Benzoyl Peroxide Acts as a Promoter of Radiation-Induced Malignant Transformation in Vitro. *Biochemical and biophysical research communications,* 212**,** 118-125.
KESKIN, Ş., ŞIRIN, Y., ÇAKIR, H. & KESKIN, M. 2019. An investigation of Humulus lupulus L.: Phenolic composition, antioxidant capacity and inhibition properties of clinically important enzymes. *South African Journal of Botany,* 120**,** 170-174.
KHODDAMI, A., WILKES, M. A. & ROBERTS, T. H. 2013. Techniques for analysis of plant phenolic compounds. *Molecules,* 18**,** 2328-2375.
KNEZ HRNČIČ, M., ŠPANINGER, E., KOŠIR, I. J., KNEZ, Ž. & BREN, U. 2019. Hop compounds: Extraction techniques, chemical analyses, antioxidative, antimicrobial, and anticarcinogenic effects. *Nutrients,* 11**,** 257.
KOBUCHI, H., VIRGILL, F. & PACKER, L. 1999. [51] Assay of inducible form of nitric oxide synthase activity: Effect of flavonoids and plant extracts. *Methods in enzymology,* 301**,** 504-513.
KÖNIG, A., SADOVA, N., DORNMAYR, M., SCHWARZINGER, B., NEUHAUSER, C., STADLBAUER, V., WALLNER, M., WOISCHITZSCHLÄGER, J., MÜLLER, A. & TONA, R. 2023. Combined acid hydrolysis and fermentation improves bioactivity of citrus flavonoids in vitro and in vivo. *Communications Biology,* 6**,** 1083.
KORNPOINTNER, C., SCHEIBELREITER, J. & HALBWIRTH, H. 2022. Snailase: a promising tool for the enzymatic hydrolysis of flavonoid glycosides from plant extracts. *Frontiers in Plant Science,* 13**,** 889184.
KOWALCZYK, D., ŚWIECA, M., CICHOCKA, J. & GAWLIK‐DZIKI, U. 2013. The phenolic content and antioxidant activity of the aqueous and hydroalcoholic extracts of hops and their pellets. *Journal of the Institute of Brewing,* 119**,** 103-110.
KROFTA, K., MIKYŠKA, A. & HAŠKOVÁ, D. 2008. Antioxidant characteristics of hops and hop products. *Journal of the Institute of Brewing,* 114**,** 160-166.
KRUEGER, C. G., REED, J. D., FELICIANO, R. P. & HOWELL, A. B. 2013. Quantifying and characterizing proanthocyanidins in cranberries in relation to urinary tract health. *Analytical and bioanalytical chemistry,* 405**,** 4385-4395.
KUMAR, S. & PANDEY, A. 2013. Chemistry and biological activity of flavanoids: An overview. 2013. *The Scientific World Journal,* 162750.
KUNEJ, U., MIKULIČ-PETKOVŠEK, M., RADIŠEK, S. & ŠTAJNER, N. 2020. Changes in the phenolic compounds of hop (Humulus lupulus L.) Induced by infection with Verticillium nonalfalfae, the causal agent of hop Verticillium wilt. *Plants,* 9**,** 841.

KURUMATANI, M., FUJITA, R., TAGASHIRA, M., SHOJI, T., KANDA, T., IKEDA, M., SHOJI, A., YANAGIDA, A., SHIBUSAWA, Y. & SHINDO, H. 2005. Analysis of polyphenols from hop bract region using CCC. *Journal of liquid chromatography & related technologies,* 28**,** 1971-1983.

LABORDE, B., MOINE-LEDOUX, V., RICHARD, T., SAUCIER, C., DUBOURDIEU, D. & MONTI, J.-P. 2006. PVPP− polyphenol complexes: a molecular approach. *Journal of Agricultural and Food Chemistry,* 54**,** 4383-4389.

LAFKA, T.-I., SINANOGLOU, V. & LAZOS, E. S. 2007. On the extraction and antioxidant activity of phenolic compounds from winery wastes. *Food chemistry,* 104**,** 1206-1214.

LAKKA, A., KARAGEORGOU, I., KALTSA, O., BATRA, G., BOZINOU, E., LALAS, S. & MAKRIS, D. 2019. Polyphenol extraction from Humulus lupulus (hop) using a neoteric glycerol/L-alanine deep eutectic solvent: Optimisation, kinetics and the effect of ultrasound-assisted pretreatment. *AgriEngineering,* 1**,** 403-417.

LAPORNIK, B., PROŠEK, M. & WONDRA, A. G. 2005. Comparison of extracts prepared from plant by-products using different solvents and extraction time. *Journal of food engineering,* 71**,** 214-222.

LEONG, C. N. A., TAKO, M., HANASHIRO, I. & TAMAKI, H. 2008. Antioxidant flavonoid glycosides from the leaves of Ficus pumila L. *Food Chemistry,* 109**,** 415-420.

LI, C., TROMBLEY, J. D., SCHMIDT, M. A. & HAGERMAN, A. E. 2010. Preparation of an acid butanol standard from fresh apples. *Journal of chemical ecology,* 36**,** 453-460.

LI, H.-J. & DEINZER, M. L. 2006. Structural identification and distribution of proanthocyanidins in 13 different hops. *Journal of Agricultural and Food Chemistry,* 54**,** 4048-4056.

LI, H.-J. & DEINZER, M. L. 2009. Proanthocyanidins in hops. *Beer in health and disease prevention.* Elsevier.

LI, Y., WANG, R., LIN, Y., HAN, B., WANG, B. & WANG, S. 2020. Qualitative and quantitative analysis of phenolic acid glycosides in Ginkgo biloba L. leaf, G. biloba leaf extract and its injection. *Biomedical Chromatography,* 34**,** e4964.

LIBRÁN CUERVAS-MONS, C. M., MAYOR LÓPEZ, L., GARCÍA CASTELLÓ, E. M. & VIDAL BROTONS, D. J. 2013. Polyphenol extraction from grape wastes: Solvent and pH effect. *Agricultural Sciences,* 4**,** 56-62.

LIN, Y.-L., JUAN, I.-M., CHEN, Y.-L., LIANG, Y.-C. & LIN, J.-K. 1996. Composition of polyphenols in fresh tea leaves and associations of their oxygen-radical-absorbing capacity with antiproliferative actions in fibroblast cells. *Journal of Agricultural and Food Chemistry,* 44**,** 1387-1394.

LINDEMANN, M., WIDHALM, B., KUNCINGER, T. & SREBOTNIK, E. 2020. An integrated process for combined microbial VOC reduction and effluent valorization in the wood processing industry. *Bioresource Technology Reports,* 11**,** 100471.

LINFORTH, R. S., WESTWOOD, K., SOMANI, A., DOHERTY, N. & COOK, D. 2015. Hop proanthocyanidins for the fining of beer. *Journal of the Institute of Brewing,* 121**,** 490-495.

LIU, C., GAO, X., LIU, Y., HUANG, M., QU, D. & CHEN, Y. 2017. Icariin combined with snailase shows improved intestinal hydrolysis and absorption in osteoporosis rats. *Biomedicine & Pharmacotherapy,* 94**,** 1048-1056.

LIU, C., LI, R., PENG, J., QU, D., HUANG, M. & CHEN, Y. 2019. Enhanced hydrolysis and antitumor efficacy of Epimedium flavonoids mediated by immobilized snailase on silica. *Process Biochemistry,* 86**,** 80-88.

LIU, M., HANSEN, P. E., WANG, G., QIU, L., DONG, J., YIN, H., QIAN, Z., YANG, M. & MIAO, J. 2015. Pharmacological profile of xanthohumol, a prenylated flavonoid from hops (Humulus lupulus). *Molecules,* 20**,** 754-779.

LIYANA-PATHIRANA, C. & SHAHIDI, F. 2005. Optimization of extraction of phenolic compounds from wheat using response surface methodology. *Food chemistry,* 93**,** 47-56.
LOOMIS, W. D. & BATTAILE, J. 1966. Plant phenolic compounds and the isolation of plant enzymes. *Phytochemistry,* 5**,** 423-438.
LORCA, F., QUINTÍN, D. & GARCÍA, P. 2023. RECOVERY OF HESPERIDIN FROM LEMON BY-PRODUCTS USING GREEN TECHNIQUES. *Food and Environment Safety Journal,* 21.
LOTITO, S. B., ACTIS-GORETTA, L., RENART, M. L., CALIGIURI, M., REIN, D., SCHMITZ, H. H., STEINBERG, F. M., KEEN, C. L. & FRAGA, C. G. 2000. Influence of oligomer chain length on the antioxidant activity of procyanidins. *Biochemical and Biophysical Research Communications,* 276**,** 945-951.
LU, H., TIAN, Z., CUI, Y., LIU, Z. & MA, X. 2020. Chlorogenic acid: A comprehensive review of the dietary sources, processing effects, bioavailability, beneficial properties, mechanisms of action, and future directions. *Comprehensive Reviews in Food Science and Food Safety,* 19**,** 3130-3158.
LUDWIG, M., DÜHRKOP, K. & BÖCKER, S. 2018. Bayesian networks for mass spectrometric metabolite identification via molecular fingerprints. *Bioinformatics,* 34**,** i333-i340.
LUZAK, B., GOLANSKI, J., PRZYGODZKI, T., BONCLER, M., SOSNOWSKA, D., OSZMIANSKI, J., WATALA, C. & ROZALSKI, M. 2016. Extract from spent hop (Humulus lupulus L.) reduces blood platelet aggregation and improves anticoagulant activity of human endothelial cells in vitro. *Journal of functional Foods,* 22**,** 257-269.
MACCHIONI, V., PICCHI, V. & CARBONE, K. 2021. Hop Leaves as an Alternative Source of Health-Active Compounds: Effect of Genotype and Drying Conditions. *Plants,* 11**,** 99.
MACCHIONI, V., PICCHI, V. & CARBONE, K. 2022. Hop Leaves as an Alternative Source of Health-Active Compounds: Effect of Genotype and Drying Conditions. *Plants,* 11**,** 99.
MAGALHÃES, P. J., VIEIRA, J. S., GONÇALVES, L. M., PACHECO, J. G., GUIDO, L. F. & BARROS, A. A. 2010. Isolation of phenolic compounds from hop extracts using polyvinylpolypyrrolidone: Characterization by high-performance liquid chromatography–diode array detection–electrospray tandem mass spectrometry. *Journal of Chromatography A,* 1217**,** 3258-3268.
MAIETTI, A., BRIGHENTI, V., BONETTI, G., TEDESCHI, P., PRENCIPE, F. P., BENVENUTI, S., BRANDOLINI, V. & PELLATI, F. 2017. Metabolite profiling of flavonols and in vitro antioxidant activity of young shoots of wild Humulus lupulus L.(hop). *Journal of Pharmaceutical and Biomedical Analysis,* 142**,** 28-34.
MALLMANN, L. P., RIOS, A. O. & RODRIGUES, E. 2023. MS-FINDER and SIRIUS for phenolic compound identification from high-resolution mass spectrometry data. *Food Research International,* 163**,** 112315.
MANASA, D., SRINIVAS, P. & SOWBHAGYA, H. 2013. Enzyme-assisted extraction of bioactive compounds from ginger (Zingiber officinale Roscoe). *Food Chemistry,* 139**,** 509-514.
MARRIOTT, R. J. 2010. Greener chemistry preparation of traditional flavour extracts and molecules. *Agro Food Industry Hi-Tech,* 21**,** 46-48.
MCCALLUM, J. L., NABUURS, M. H., GALLANT, S. T. & KIRBY, C. W. 2019. Phytochemical characterization of wild hops (Humulus lupulus ssp. lupuloides) germplasm resources from the maritimes region of Canada. *Frontiers in plant science,* 10**,** 479751.
MCMURROUGH, I., MADIGAN, D. & SMYTH, M. R. 1995. Adsorption by polyvinylpolypyrrolidone of catechins and proanthocyanidins from beer. *Journal of Agricultural and Food Chemistry,* 43**,** 2687-2691.
MENDES, D., OLIVEIRA, M. M., MOREIRA, P. I., COUTINHO, J., NUNES, F. M., PEREIRA, D. M., VALENTÃO, P., ANDRADE, P. B. & VIDEIRA, R. A. 2018. Beneficial effects of white wine polyphenols-enriched diet on Alzheimer's disease-like pathology. *The Journal of Nutritional Biochemistry,* 55**,** 165-177.

MERTENS, T., KUNZ, T. & GIBSON, B. R. 2022. Transition metals in brewing and their role in wort and beer oxidative stability: a review. *Journal of the Institute of Brewing,* 128**,** 77-95.
MERTENS, T., KUNZ, T., WIETSTOCK, P. C. & METHNER, F. J. 2021. Complexation of transition metals by chelators added during mashing and impact on beer stability. *Journal of the Institute of Brewing,* 127**,** 345-357.
MIKŠOVSKY, P., KORNPOINTNER, C., PARANDEH, Z., GOESSINGER, M., BICA-SCHRÖDER, K. & HALBWIRTH, H. 2023. Enzyme‐Assisted Supercritical Fluid Extraction of Flavonoids from Apple Pomace (Malus× domestica). *ChemSusChem***,** e202301094.
MIKYŠKA, A., JURKOVÁ, M., HORÁK, T. & SLABÝ, M. 2022. Study of the influence of hop polyphenols on the sensory stability of lager beer. *European Food Research and Technology,* 248**,** 533-542.
MIKYŠKA, A. & KROFTA, K. 2012. Assessment of changes in hop resins and polyphenols during long‐term storage. *Journal of the Institute of Brewing,* 118**,** 269-279.
MIKYŠKA, A., KROFTA, K., HOUŠKA, M. & ČERMÁK, P. Pascalization, alternative way of postharvest hops processing. IV International Humulus Symposium 1236, 2015. 23-30.
MIR-CERDÀ, A., NUNEZ, O., GRANADOS, M., SENTELLAS, S. & SAURINA, J. 2023. An overview of the extraction and characterization of bioactive phenolic compounds from agri-food waste within the framework of circular bioeconomy. *TrAC Trends in Analytical Chemistry,* 161**,** 116994.
MIR-CERDÀ, A., RAMOS-CALERO, V., BELTRÁN, J. L., CORTINA, J. L., SAURINA, J., SENTELLAS, S. & GRANADOS, M. 2024. Towards a sustainable recovery of polyphenols from agrifood waste: performance of polymeric sorbents with natural deep eutectic solvent extracts. *LWT***,** 116632.
MIROWSKI, J., OLIWA, R., OLEKSY, M., RÓJ, E., TOMASZEWSKA, J., MIZERA, K. & RYSZKOWSKA, J. 2021. Composites of Poly (vinyl chloride) with Residual Hops after Supercritical Extraction in CO2. Polymers 2021, 13, 2736. s Note: MDPI stays neutral with regard to jurisdictional claims in published ….
MISHRA, A. K., KOCÁBEK, T., SUKUMARI NATH, V., AWASTHI, P., SHRESTHA, A., KUMAR KILLI, U., JAKSE, J., PATZAK, J., KROFTA, K. & MATOUŠEK, J. 2020. Dissection of dynamic transcriptome landscape of leaf, bract, and lupulin gland in hop (Humulus lupulus L.). *International journal of molecular sciences,* 21**,** 233.
MOENS, E., BOLCA, S., VAN DE WIELE, T., VAN LANDSCHOOT, A., GOEMAN, J. L., POSSEMIERS, S. & VERSTRAETE, W. 2020. Exploration of isoxanthohumol bioconversion from spent hops into 8-prenylnaringenin using resting cells of Eubacterium limosum. *AMB Express,* 10**,** 1-11.
MOHARRAM, H. & YOUSSEF, M. 2014. Methods for determining the antioxidant activity: A review. *Alexandria Journal of Food Science and Technology,* 11**,** 31-42.
MOHD ABD RAZAK, M. R., NORAHMAD, N. A., MD JELAS, N. H., AFZAN, A., MOHMAD MISNAN, N., MAT RIPEN, A., THAYAN, R., ZAINOL, M. & SYED MOHAMED, A. F. 2021. Immunomodulatory activities of Carica papaya L. leaf juice in a non-lethal, symptomatic dengue mouse model. *Pathogens,* 10**,** 501.
MORCOL, T. B., MATTHEWS, P. D. & KENNELLY, E. J. 2021. Differences in Leaf Chemistry and Glandular Trichome Density between Wild Southwestern American Hop (Humulus neomexicanus) and Commercial Hop Cultivars. *Journal of Agricultural and Food Chemistry,* 69**,** 7798-7814.
MORCOL, T. B., WYSOCKI, K., SANKARAN, R. P., MATTHEWS, P. D. & KENNELLY, E. J. 2020. UPLC-QTof-MSE Metabolomics Reveals Changes in Leaf Primary and Secondary Metabolism of Hop (Humulus lupulus L.) Plants under Drought Stress. *Journal of Agricultural and Food Chemistry,* 68**,** 14698-14708.

MORDOR INTELLIGENCE. n.d. *POLYPHENOL MARKET SIZE & SHARE ANALYSIS - GROWTH TRENDS & FORECASTS (2023 - 2028)* [Online].). [Accessed].
MUZYKIEWICZ, A., NOWAK, A., ZIELONKA-BRZEZICKA, J., FLORKOWSKA, K., DUCHNIK, W. & KLIMOWICZ, A. 2019. Comparison of antioxidant activity of extracts of hop leaves harvested in different years. *Herba Polonica,* 65.
MYO, H. & KHAT-UDOMKIRI, N. 2022. Optimization of ultrasound-assisted extraction of bioactive compounds from coffee pulp using propylene glycol as a solvent and their antioxidant activities. *Ultrasonics Sonochemistry,* 89**,** 106127.
NADAR, S. S., RAO, P. & RATHOD, V. K. 2018. Enzyme assisted extraction of biomolecules as an approach to novel extraction technology: A review. *Food Research International,* 108**,** 309-330.
NAGEL, J., CULLEY, L. K., LU, Y., LIU, E., MATTHEWS, P. D., STEVENS, J. F. & PAGE, J. E. 2008. EST analysis of hop glandular trichomes identifies an O-methyltransferase that catalyzes the biosynthesis of xanthohumol. *The Plant Cell,* 20**,** 186-200.
NATELLA, F., NARDINI, M., DI FELICE, M. & SCACCINI, C. 1999. Benzoic and cinnamic acid derivatives as antioxidants: Structure− activity relation. *Journal of agricultural and food chemistry,* 47**,** 1453-1459.
NAVARRO, M., ZAMORA, W., QUESADA, S., AZOFEIFA, G., ALVARADO, D. & MONAGAS, M. 2017. Fractioning of proanthocyanidins of Uncaria tomentosa. Composition and structure-bioactivity relationship. *Antioxidants,* 6**,** 60.
NAYAK, A., BHUSHAN, B., ROSALES, A., TURIENZO, L. R. & CORTINA, J. 2018. Valorisation potential of Cabernet grape pomace for the recovery of polyphenols: Process intensification, optimisation and study of kinetics. *Food and Bioproducts Processing,* 109**,** 74-85.
NESVADBA, V., OLŠOVSKÁ, J., STRAKOVÁ, L. & CHARVÁTOVÁ, J. 2021. Evaluation of Czech hop varieties. *KVASNY PRUMYSL,* 67**,** 428-434.
NICACIO, K. D. J., FERREIRA, M. S., KATCHBORIAN‐NETO, A., COSTA, M. L., MURGU, M., DIAS, D. F., SOARES, M. G. & CHAGAS‐PAULA, D. A. 2022. Anti‐Inflammatory Markers of Hops Cultivars (Humulus lupulus L.) Evaluated by Untargeted Metabolomics Strategy. *Chemistry & Biodiversity,* 19**,** e202100966.
NOORDMAN, T. R., VAN DER NOORDT, M. & RICHTER, A. 2016. Method for the regeneration of PVPP from a membrane filter retentate after clarification and stabilization of a yeast fermented beverage. Google Patents.
NOUR, V., PANAITE, T. D., CORBU, A. R., ROPOTA, M. & TURCU, R. P. 2021. Nutritional and Bioactive Compounds in Dried Sea-Buckthorn Pomace. *Erwerbs-Obstbau,* 63.
NOWAK, R., OLECH, M. & NOWACKA, N. 2014. Plant polyphenols as chemopreventive agents. *Polyphenols in human health and disease***,** 1289-1307.
NUNES, M. A., RODRIGUES, F. & OLIVEIRA, M. B. P. 2017. Grape processing by-products as active ingredients for cosmetic proposes. *Handbook of grape processing by-products.* Elsevier.
OLIVARES-GALVÁN, S., MARINA, M. & GARCÍA, M. 2022. Extraction of valuable compounds from brewing residues: Malt rootlets, spent hops, and spent yeast. *Trends in Food Science & Technology,* 127**,** 181-197.
OLŠOVSKÁ, J., KAMENÍK, Z., ČEJKA, P., JURKOVÁ, M. & MIKYŠKA, A. 2013. Ultra-high-performance liquid chromatography profiling method for chemical screening of proanthocyanidins in Czech hops. *Talanta,* 116**,** 919-926.
ÖNDER, F. C. M., AY, M. & SARKER, S. D. 2013. Comparative study of antioxidant properties and total phenolic content of the extracts of Humulus lupulus L. and quantification of bioactive components by LC–MS/MS and GC–MS. *Journal of agricultural and food chemistry,* 61**,** 10498-10506.

OSORIO, L. L. D. R., FLÓREZ-LÓPEZ, E. & GRANDE-TOVAR, C. D. 2021. The potential of selected agri-food loss and waste to contribute to a circular economy: Applications in the food, cosmetic and pharmaceutical industries. *Molecules,* 26**,** 515.

OU, B., HAMPSCH-WOODILL, M., FLANAGAN, J., DEEMER, E. K., PRIOR, R. L. & HUANG, D. 2002. Novel fluorometric assay for hydroxyl radical prevention capacity using fluorescein as the probe. *Journal of agricultural and food chemistry,* 50**,** 2772-2777.

OU, B., HAMPSCH-WOODILL, M. & PRIOR, R. L. 2001. Development and validation of an improved oxygen radical absorbance capacity assay using fluorescein as the fluorescent probe. *Journal of agricultural and food chemistry,* 49**,** 4619-4626.

PACKER, L., RIMBACH, G. & VIRGILI, F. 1999. Antioxidant activity and biologic properties of a procyanidin-rich extract from pine (Pinus maritima) bark, pycnogenol. *Free Radical Biology and Medicine,* 27**,** 704-724.

PALMIOLI, A., MAZZONI, V., DE LUIGI, A., BRUZZONE, C., SALA, G., COLOMBO, L., BAZZINI, C., ZOIA, C. P., INSERRA, M. & SALMONA, M. 2022. Alzheimer's Disease Prevention through Natural Compounds: Cell-Free, In Vitro, and In Vivo Dissection of Hop (Humulus lupulus L.) Multitarget Activity. *ACS Chemical Neuroscience,* 13**,** 3152-3167.

PANAT, N. A., AMRUTE, B. K., BHATTU, S., HARAM, S. K., SHARMA, G. K. & GHASKADBI, S. S. 2015. Antioxidant Profiling of C3 Quercetin Glycosides: Quercitrin, Quercetin 3-β-D-glucoside and Quercetin 3-O-(6"-O-malonyl)-β-Dglucoside in cell free environment. *Free Radicals and Antioxidants,* 5**,** 90-100.

PAREDES-RAMOS, M., CONDE PIÑEIRO, E. & LOPEZ VILARIÑO, J. M. 2022. Brewers' spent hop revalorization for the production of high added-value cosmetics ingredients with elastase inhibition capacity. *Scientific Reports,* 12**,** 22074.

PARK, J. S., RHO, H. S., KIM, D. H. & CHANG, I. S. 2006. Enzymatic preparation of kaempferol from green tea seed and its antioxidant activity. *Journal of agricultural and food chemistry,* 54**,** 2951-2956.

PATZAK, J., KROFTA, K., HENYCHOVÁ, A. & NESVADBA, V. 2015. Number and size of lupulin glands, glandular trichomes of hop (Humulus lupulus L.), play a key role in contents of bitter acids and polyphenols in hop cone. *International Journal of Food Science & Technology,* 50**,** 1864-1872.

PAUNOVIĆ, D. Đ., MITIĆ, S. S., STOJANOVIĆ, G. S., MITIĆ, M. N., STOJANOVIĆ, B. T. & STOJKOVIĆ, M. B. 2015. Kinetics of the solid-liquid extraction process of phenolic antioxidants and antioxidant capacity from hop (Humulus lupulus L.). *Separation Science and Technology,* 50**,** 1658-1664.

PAYNE, M. J., HURST, W. J., STUART, D. A., OU, B., FAN, E., JI, H. & KOU, Y. 2010. Determination of total procyanidins in selected chocolate and confectionery products using DMAC. *Journal of AOAC International,* 93**,** 89-96.

PEREIRA, O. R., SANTOS, G. & SOUSA, M. J. 2022. Hop by-products: Pharmacological activities and potential application as cosmetics. *Cosmetics,* 9**,** 139.

PÉREZ-LARRÁN, P., DÍAZ-REINOSO, B., MOURE, A., ALONSO, J. L. & DOMÍNGUEZ, H. 2018. Adsorption technologies to recover and concentrate food polyphenols. *Current Opinion in Food Science,* 23**,** 165-172.

PINELO, M., TRESS, A. G., PEDERSEN, M., ARNOUS, A. & MEYER, A. S. 2007. Effect of cellulases, solvent type and particle size distribution on the extraction of chlorogenic acid and other phenols from spent coffee grounds. *Am. J. Food Technol,* 2**,** 641-651.

PLATZER, M., KIESE, S., HERFELLNER, T., SCHWEIGGERT-WEISZ, U. & EISNER, P. 2021. How does the phenol structure influence the results of the Folin-Ciocalteu assay? *Antioxidants,* 10**,** 811.

PLUMB, G. W., DE PASCUAL-TERESA, S., SANTOS-BUELGA, C., CHEYNIER, V. & WILLIAMSON, G. 1998. Antioxidant properties of catechins and proanthocyanidins: Effect of polymerisation, galloylation and glycosylation. *Free radical research,* 29**,** 351-358.

POSSEMIERS, S., BOLCA, S., GROOTAERT, C., HEYERICK, A., DECROOS, K., DHOOGE, W., DE KEUKELEIRE, D., RABOT, S., VERSTRAETE, W. & VAN DE WIELE, T. 2006. The prenylflavonoid isoxanthohumol from hops (Humulus lupulus L.) is activated into the potent phytoestrogen 8-prenylnaringenin in vitro and in the human intestine. *The Journal of nutrition,* 136**,** 1862-1867.

PROESTOS, C. & KOMAITIS, M. 2009. Antioxidant capacity of hops. *Beer in health and disease prevention.* Elsevier.

PŠENÁKOVÁ, I., HETEŠOVÁ, L., NEMECEK, P., FARAGÓ, J. & KRAIC, J. 2010. GENOTYPE AND SEASONAL VARIATION IN ANTIOXIDANT ACTIVITY OF HOP EXTRACTS. *Agriculture/Pol'nohospodárstvo,* 56.

PULIDO, R., BRAVO, L. & SAURA-CALIXTO, F. 2000. Antioxidant activity of dietary polyphenols as determined by a modified ferric reducing/antioxidant power assay. *Journal of agricultural and food chemistry,* 48**,** 3396-3402.

QIAN, J., ZHU, H., ZHANG, J., ZHAO, C., LI, X. & GUO, H. 2023. Separation and Purification of Bamboo Leaf Flavones by Polyvinylpolypyrrolidone Adsorption. *Journal of Chromatographic Science,* 61**,** 885-891.

RAO, P. V. & GAN, S. H. 2014. Cinnamon: a multifaceted medicinal plant. *Evidence-Based Complementary and Alternative Medicine,* 2014.

RAȚU, R. N., VELEȘCU, I. D., STOICA, F., USTUROI, A., ARSENOAIA, V. N., CRIVEI, I. C., POSTOLACHE, A. N., LIPȘA, F. D., FILIPOV, F. & FLOREA, A. M. 2023. Application of Agri-food by-products in the food industry. *Agriculture,* 13**,** 1559.

RATZ‐ŁYKO, A., ARCT, J., MAJEWSKI, S. & PYTKOWSKA, K. 2015. Influence of polyphenols on the physiological processes in the skin. *Phytotherapy Research,* 29**,** 509-517.

RAVBER, M., KNEZ, Ž. & ŠKERGET, M. 2015. Optimization of hydrolysis of rutin in subcritical water using response surface methodology. *The Journal of Supercritical Fluids,* 104**,** 145-152.

REID, N., GOETZ, S. J., DOBIS, E. A., GOTTLIEB, P. D. & HIRA, A. 2020. The impact of the craft beer revolution on the American hop industry. *Rural-Urban Linkages for Sustainable Development.* Routledge.

RENAUD, S. D. & DE LORGERIL, M. 1992. Wine, alcohol, platelets, and the French paradox for coronary heart disease. *The Lancet,* 339**,** 1523-1526.

RIBEIRO, M. H. L. 2015. Glycosides: From biosynthesis to biological activity toward therapeutic application. *Biotechnology of Bioactive Compounds: Sources and Applications***,** 301-344.

ROBERTS, T. & WILSON, R. 2006. Handbook of brewing, –. *Boca Raton: Hops. Taylor & Francis***,** 177-280.

ROBERTS, T. R. 2016. Hops. *Brewing Materials and Processes.* Elsevier.

ROCHA, L., NEVES, D., VALENTÃO, P., ANDRADE, P. B. & VIDEIRA, R. A. 2020. Adding value to polyvinylpolypyrrolidone winery residue: A resource of polyphenols with neuroprotective effects and ability to modulate type 2 diabetes-relevant enzymes. *Food chemistry,* 329**,** 127168.

RODOLFI, M., BARBANTI, L., GIORDANO, C., RINALDI, M., FABBRI, A., PRETTI, L., CASOLARI, R., BEGHÉ, D., PETRUCCELLI, R. & GANINO, T. 2021. The Effect of Different Organic Foliar Fertilization on Physiological and Chemical Characters in Hop (Humulus lupulus L., cv Cascade) Leaves and Cones. *Applied Sciences,* 11**,** 6778.

ROUTRAY, W. & ORSAT, V. 2019. Agricultural and food industry by-products: source of bioactive components for functional beverages. *Nutrients in beverages.* Elsevier.

RÓZEK, A., GARCÍA-PÉREZ, J. V., LÓPEZ, F., GÜELL, C. & FERRANDO, M. 2010. Infusion of grape phenolics into fruits and vegetables by osmotic treatment: Phenolic stability during air drying. *Journal of food engineering,* 99**,** 142-150.

RUIZ-RUIZ, J. C., ALDANA, G. D. C. E., CRUZ, A. I. C. & SEGURA-CAMPOS, M. R. 2020. Antioxidant activity of polyphenols extracted from hop used in craft beer. *Biotechnological Progress and Beverage Consumption.* Elsevier.

SAHA, N., BALAKRISHNAN, M. & ULBRICHT, M. 2006. Polymeric membrane fouling in sugarcane juice ultrafiltration: role of juice polysaccharides. *Desalination,* 189**,** 59-70.

ŠAMEC, D., KARALIJA, E., ŠOLA, I., VUJČIĆ BOK, V. & SALOPEK-SONDI, B. 2021. The role of polyphenols in abiotic stress response: The influence of molecular structure. *Plants,* 10**,** 118.

SÁNCHEZ-RANGEL, J. C., BENAVIDES, J., HEREDIA, J. B., CISNEROS-ZEVALLOS, L. & JACOBO-VELÁZQUEZ, D. A. 2013. The Folin–Ciocalteu assay revisited: improvement of its specificity for total phenolic content determination. *Analytical methods,* 5**,** 5990-5999.

SANTAGOSTINI, L., CAPORALI, E., GIULIANI, C., BOTTONI, M., ASCRIZZI, R., ARANEO, S. R., PAPINI, A., FLAMINI, G. & FICO, G. 2020. Humulus lupulus L. cv. Cascade grown in Northern Italy: morphological and phytochemical characterization. *Plant Biosystems-An International Journal Dealing with all Aspects of Plant Biology,* 154**,** 316-325.

SANTAMARÍA, B., SALAZAR, G., BELTRÁN, S. & CABEZAS, J. 2002. Membrane sequences for fractionation of polyphenolic extracts from defatted milled grape seeds. *Desalination,* 148**,** 103-109.

SANTOS, C. M. & SILVA, A. M. 2020. The antioxidant activity of prenylflavonoids. *Molecules,* 25**,** 696.

SANZ, V., TORRES, M. D., VILARIÑO, J. M. L. & DOMÍNGUEZ, H. 2019. What is new on the hop extraction? *Trends in Food Science & Technology,* 93**,** 12-22.

SARRAF, C., DESJARDINS, Y., LEONHART, S., GOSSELIN, A. & GOSSELIN, G. Agronomic and nutraceutical potential of hops (Humulus lupulus L.) grown in Québec, Canada. III International Humulus Symposium 1010, 2012. 155-161.

SCHMIDT, C. & BIENDL, M. 2023a. Quantitative analysis of a large spectrum of hop phenolic compounds by LC-MS/MS. *Brewing Science,* 76.

SCHMIDT, C. & BIENDL, M. 2023b. Quantitative analysis of a large spectrum of hop phenolic compounds by LC-MS/MS.

SCHMIDT, R., SCHULMEYR, J. & GEHRIG, M. Production of xanthohumol enriched hop extract using carbon dioxide as solvent at pressures up to 1000 bars. Proceedings of the Scientific Commission of the International Hop Growers Convention, South Africa, 2005.

SCHÖNBERGER, C. 2006. The processing of hops. *Brewing.* Elsevier.

SCHÖNBERGER, C. & KOSTELECKY, T. 2011. 125th anniversary review: The role of hops in brewing. *Journal of the Institute of Brewing,* 117**,** 259-267.

SCHUDDEBOOM, L. 1993. *Nitrates and nitrites in foodstuffs*, Council of Europe.

SCOMA, A., PINTUCCI, C., BERTIN, L., CARLOZZI, P. & FAVA, F. 2012. Increasing the large scale feasibility of a solid phase extraction procedure for the recovery of natural antioxidants from olive mill wastewaters. *Chemical Engineering Journal,* 198**,** 103-109.

SHAHIDI, F., VARATHARAJAN, V., OH, W. Y. & PENG, H. 2019. Phenolic compounds in agri-food by-products, their bioavailability and health effects. *Food Bioact,* 5**,** 57-119.

SHARMA, O. P. & BHAT, T. K. 2009. DPPH antioxidant assay revisited. *Food chemistry,* 113**,** 1202-1205.

SHEN, H., DÜHRKOP, K., BÖCKER, S. & ROUSU, J. 2014. Metabolite identification through multiple kernel learning on fragmentation trees. *Bioinformatics,* 30**,** i157-i164.
SHI, J., YU, J., POHORLY, J., YOUNG, J. C., BRYAN, M. & WU, Y. 2003. Optimization of the extraction of polyphenols from grape seed meal by aqueous ethanol solution. *J. Food Agric. Environ,* 1**,** 42-47.
SHIMODA, K., OTSUKA, T., MORIMOTO, Y., HAMADA, H. & HAMADA, H. 2007. Glycosylation and malonylation of quercetin, epicatechin, and catechin by cultured plant cells. *Chemistry letters,* 36**,** 1292-1293.
SHINADA, K., TAGASHIRA, M., WATANABE, H., SOPAPORNAMORN, P., KANAYAMA, A., KANDA, T., IKEDA, M. & KAWAGUCHI, Y. 2007. Hop bract polyphenols reduced three-day dental plaque regrowth. *Journal of dental research,* 86**,** 848-851.
SILVA, K. F. C. E., STRIEDER, M. M., PINTO, M. B. C., ROSTAGNO, M. A. & HUBINGER, M. D. 2023. Processing Strategies for Extraction and Concentration of Bitter Acids and Polyphenols from Brewing By-Products: A Comprehensive Review. *Processes,* 11**,** 921.
SINGLETON, V. L., ORTHOFER, R. & LAMUELA-RAVENTÓS, R. M. 1999. [14] Analysis of total phenols and other oxidation substrates and antioxidants by means of folin-ciocalteu reagent. *Methods in enzymology.* Elsevier.
SOLOMAKOU, N., LOUKRI, A., TSAFRAKIDOU, P., MICHAELIDOU, A.-M., MOURTZINOS, I. & GOULA, A. M. 2022. Recovery of phenolic compounds from spent coffee grounds through optimized extraction processes. *Sustainable Chemistry and Pharmacy,* 25**,** 100592.
SPRENG, S. & HOFMANN, T. 2018. Activity-guided identification of in vitro antioxidants in beer. *Journal of agricultural and food chemistry,* 66**,** 720-731.
STANIUS, Ž., DŪDĖNAS, M., KAŠKONIENĖ, V., STANKEVIČIUS, M., SKRZYDLEWSKA, E., DREVINSKAS, T., RAGAŽINSKIENĖ, O., OBELEVIČIUS, K. & MARUŠKA, A. 2022. Analysis of the Leaves and Cones of Lithuanian Hops (Humulus lupulus L.) Varieties by Chromatographic and Spectrophotometric Methods. *Molecules,* 27**,** 2705.
STEVENS, J. F., IVANCIC, M., HSU, V. L. & DEINZER, M. L. 1997. Prenylflavonoids from Humulus lupulus. *Phytochemistry,* 44**,** 1575-1585.
STEVENS, J. F., MIRANDA, C. L., WOLTHERS, K. R., SCHIMERLIK, M., DEINZER, M. L. & BUHLER, D. R. 2002. Identification and in vitro biological activities of hop proanthocyanidins: inhibition of nNOS activity and scavenging of reactive nitrogen species. *Journal of Agricultural and Food Chemistry,* 50**,** 3435-3443.
STEVENS, J. F. & PAGE, J. E. 2004. Xanthohumol and related prenylflavonoids from hops and beer: to your good health! *Phytochemistry,* 65**,** 1317-1330.
STEVENS, J. F., TAYLOR, A. W., NICKERSON, G. B., IVANCIC, M., HENNING, J., HAUNOLD, A. & DEINZER, M. L. 2000. Prenylflavonoid variation in Humulus lupulus: distribution and taxonomic significance of xanthogalenol and 4′ -O-methylxanthohumol. *Phytochemistry,* 53**,** 759-775.
SUGIYAMA, M., KATSUBE, T., KOYAMA, A. & ITAMURA, H. 2013. Varietal differences in the flavonol content of mulberry (Morus spp.) leaves and genetic analysis of quercetin 3-(6-malonylglucoside) for component breeding. *Journal of agricultural and food chemistry,* 61**,** 9140-9147.
SUN, S., WANG, X., YUAN, A., LIU, J., LI, Z., XIE, D., ZHANG, H., LUO, W., XU, H. & LIU, J. 2022. Chemical constituents and bioactivities of hops (Humulus lupulus L.) and their effects on beer‐related microorganisms. *Food and Energy Security***,** e367.
SYTAR, O., ZIVCAK, M., BRUCKOVA, K., BRESTIC, M., HEMMERICH, I., RAUH, C. & SIMKO, I. 2018. Shift in accumulation of flavonoids and phenolic acids in lettuce attributable to changes in ultraviolet radiation and temperature. *Scientia Horticulturae,* 239**,** 193-204.

TAGASHIRA, M., UCHIYAMA, K., YOSHIMURA, T., SHIROTA, M. & UEMITSU, N. 1997. Inhibition by hop bract polyphenols of cellular adherence and water-insoluble glucan synthesis of mutans streptococci. *Bioscience, biotechnology, and biochemistry,* 61**,** 332-335.

TAYLOR, A. W., BAROFSKY, E., KENNEDY, J. A. & DEINZER, M. L. 2003. Hop (Humulus lupulus L.) proanthocyanidins characterized by mass spectrometry, acid catalysis, and gel permeation chromatography. *Journal of agricultural and food chemistry,* 51**,** 4101-4110.

TING, P. L. & RYDER, D. S. 2017. The bitter, twisted truth of the hop: 50 years of hop chemistry. *Journal of the American Society of Brewing Chemists,* 75**,** 161-180.

TOŠOVIĆ, J., MARKOVIĆ, S., MARKOVIĆ, J. M. D., MOJOVIĆ, M. & MILENKOVIĆ, D. 2017. Antioxidative mechanisms in chlorogenic acid. *Food chemistry,* 237**,** 390-398.

URGEOVA, E. & POLIVKA, L. 2009. Secondary metabolites with antibacterial effects from leaves of different hop cultivars during vegetal periods. *Nova Biotechnologica,* 9**,** 327-332.

VAN HOYWEGHEN, L., BIENDL, M. & HEYERICK, A. 2010. Radical scavenging capacity of hop-derived products. *Brewing Science,* 63**,** 1-5.

VELOT, É., DUCROCQ, F., GIRARDEAU, L., HEHN, A., PIUTTI, S., KAHN, C., LINDER, M., BIANCHI, A. & ARAB-TEHRANY, E. 2022. Hop Extract Anti-Inflammatory Effect on Human Chondrocytes Is Potentiated When Encapsulated in Rapeseed Lecithin Nanoliposomes. *International Journal of Molecular Sciences,* 23**,** 12423.

VUOLO, M. M., LIMA, V. S. & JUNIOR, M. R. M. 2019. Phenolic compounds: Structure, classification, and antioxidant power. *Bioactive compounds.* Elsevier.

WANG, J., FANG, X., GE, L., CAO, F., ZHAO, L., WANG, Z. & XIAO, W. 2018a. Antitumor, antioxidant and anti-inflammatory activities of kaempferol and its corresponding glycosides and the enzymatic preparation of kaempferol. *PLoS One,* 13**,** e0197563.

WANG, J., WANG, M., WU, F., CHEN, Z.-L. & CUI, S.-M. 2012. Directionally enzymatic hydrolysis of rutin for biosynthesis of quercetin. *J. Med. Plants Res,* 6**,** 1130-1139.

WANG, J., ZHAO, L.-L., SUN, G.-X., LIANG, Y., WU, F.-A., CHEN, Z. & CUI, S. 2011. A comparison of acidic and enzymatic hydrolysis of rutin. *African Journal of Biotechnology,* 10**,** 1460-1466.

WANG, L., BOUSSETTA, N., LEBOVKA, N. & VOROBIEV, E. 2018b. Effects of ultrasound treatment and concentration of ethanol on selectivity of phenolic extraction from apple pomace. *International Journal of Food Science & Technology,* 53**,** 2104-2109.

WANG, X., YANG, L., YANG, X. & TIAN, Y. 2014. In vitro and in vivo antioxidant and antimutagenic activities of polyphenols extracted from hops (Humulus lupulus L.). *Journal of the Science of Food and Agriculture,* 94**,** 1693-1700.

WANG, Z., LI, S., GE, S. & LIN, S. 2020. Review of distribution, extraction methods, and health benefits of bound phenolics in food plants. *Journal of Agricultural and Food Chemistry,* 68**,** 3330-3343.

WANG, Z., ZHAO, L.-C., LI, W., ZHANG, L.-X., ZHANG, J. & LIANG, J. 2013. Highly efficient biotransformation of polydatin to resveratrol by snailase hydrolysis using response surface methodology optimization. *Molecules,* 18**,** 9717-9726.

WANNENMACHER, J., COTTERCHIO, C., SCHLUMBERGER, M., REUBER, V., GASTL, M. & BECKER, T. 2019. Technological influence on sensory stability and antioxidant activity of beers measured by ORAC and FRAP. *Journal of the Science of Food and Agriculture,* 99**,** 6628-6637.

WEBER, H. A., HODGES, A. E., GUTHRIE, J. R., O'BRIEN, B. M., ROBAUGH, D., CLARK, A. P., HARRIS, R. K., ALGAIER, J. W. & SMITH, C. S. 2007. Comparison of proanthocyanidins in commercial antioxidants: grape seed and pine bark extracts. *Journal of Agricultural and Food Chemistry,* 55**,** 148-156.

WEBER, N., BIEHLER, K., SCHWABE, K., HAARHAUS, B., QUIRIN, K.-W., FRANK, U., SCHEMPP, C. M. & WÖLFLE, U. 2019. Hop extract acts as an antioxidant with antimicrobial effects against Propionibacterium acnes and Staphylococcus aureus. *Molecules,* 24**,** 223.

WEREMFO, A., ABASSAH‐OPPONG, S., ADULLEY, F., DABIE, K. & SEIDU‐LARRY, S. 2023. Response surface methodology as a tool to optimize the extraction of bioactive compounds from plant sources. *Journal of the Science of Food and Agriculture,* 103**,** 26-36.

WINKEL-SHIRLEY, B. 2002. Biosynthesis of flavonoids and effects of stress. *Current opinion in plant biology,* 5**,** 218-223.

XI, J., SHEN, D., ZHAO, S., LU, B., LI, Y. & ZHANG, R. 2009. Characterization of polyphenols from green tea leaves using a high hydrostatic pressure extraction. *International journal of pharmaceutics,* 382**,** 139-143.

XIAO, F., XU, T., LU, B. & LIU, R. 2020. Guidelines for antioxidant assays for food components. *Food Frontiers,* 1**,** 60-69.

XIAO, Z., HE, L., HOU, X., WEI, J., MA, X., GAO, Z., YUAN, Y., XIAO, J., LI, P. & YUE, T. 2021. Relationships between structure and antioxidant capacity and activity of glycosylated flavonols. *Foods,* 10**,** 849.

XIE, L., DENG, Z., ZHANG, J., DONG, H., WANG, W., XING, B. & LIU, X. 2022. Comparison of flavonoid O-glycoside, C-glycoside and their aglycones on antioxidant capacity and metabolism during in vitro digestion and in vivo. *Foods,* 11**,** 882.

XU, L., KUMAR, A., LAMB, K. & LAYTON, L. 2005. Production of an isoflavone concentrate from red clover flowers. *Journal of food science,* 70**,** s558-s562.

YAMAGUCHI, N., SATOH-YAMAGUCHI, K. & ONO, M. 2009. In vitro evaluation of antibacterial, anticollagenase, and antioxidant activities of hop components (Humulus lupulus) addressing acne vulgaris. *Phytomedicine,* 16**,** 369-376.

YANAGIDA, A., SHOJI, T. & SHIBUSAWA, Y. 2003. Separation of proanthocyanidins by degree of polymerization by means of size-exclusion chromatography and related techniques. *Journal of biochemical and biophysical methods,* 56**,** 311-322.

YANG, B., LIU, H., YANG, J., GUPTA, V. K. & JIANG, Y. 2018. New insights on bioactivities and biosynthesis of flavonoid glycosides. *Trends in food science & technology,* 79**,** 116-124.

YANG, Q., ZHAO, M. & LIN, L. 2016. Adsorption and desorption characteristics of adlay bran free phenolics on macroporous resins. *Food Chemistry,* 194**,** 900-907.

YANGUI, A., NJIMOU, J. R., CICCI, A., BRAVI, M., ABDERRABBA, M. & CHIANESE, A. 2017. Competitive adsorption, selectivity and separation of valuable hydroxytyrosol and toxic phenol from olive mill wastewater. *Journal of environmental chemical engineering,* 5**,** 3581-3589.

YORDI, E. G., PÉREZ, E. M., MATOS, M. J. & VILLARES, E. U. 2012. Antioxidant and pro-oxidant effects of polyphenolic compounds and structure-activity relationship evidence. *Nutrition, well-being and health,* 2**,** 23-48.

ZAGKLIS, D. P. & PARASKEVA, C. A. 2015. Purification of grape marc phenolic compounds through solvent extraction, membrane filtration and resin adsorption/desorption. *Separation and Purification Technology,* 156**,** 328-335.

ZAM, W., BASHOUR, G., ABDELWAHED, W. & KHAYATA, W. 2012. Separation and purification of proanthocyanidins extracted from pomegranate's peels (Punica granatum). *International Journal of Pharmaceutical Sciences and Nanotechnology,* 5**,** 1808-1813.

ZANOLI, P. & ZAVATTI, M. 2008. Pharmacognostic and pharmacological profile of Humulus lupulus L. *Journal of ethnopharmacology,* 116**,** 383-396.

ZHANG, X.-L., ZHANG, Y.-D., WANG, T., GUO, H.-Y., LIU, Q.-M. & SU, H.-X. 2014. Evaluation on antioxidant effect of xanthohumol by different antioxidant capacity analytical methods. *Journal of Chemistry,* 2014.

ZHENG, H.-Z., HWANG, I.-W. & CHUNG, S.-K. 2009. Enhancing polyphenol extraction from unripe apples by carbohydrate-hydrolyzing enzymes. *Journal of Zhejiang University Science B,* 10**,** 912-919.

ZHOU, H.-C., TAM, N. F.-Y., LIN, Y.-M., DING, Z.-H., CHAI, W.-M. & WEI, S.-D. 2014. Relationships between degree of polymerization and antioxidant activities: A study on proanthocyanidins from the leaves of a medicinal mangrove plant Ceriops tagal. *PLoS One,* 9**,** e107606.

ZHU, H., LI, P., REN, S., TAN, W. & FANG, G. 2019. Low-cost Ru/C-catalyzed depolymerization of the polymeric proanthocyanidin-rich fraction from bark to produce oligomeric proanthocyanidins with antioxidant activity. *Acs Omega,* 4**,** 16471-16480.

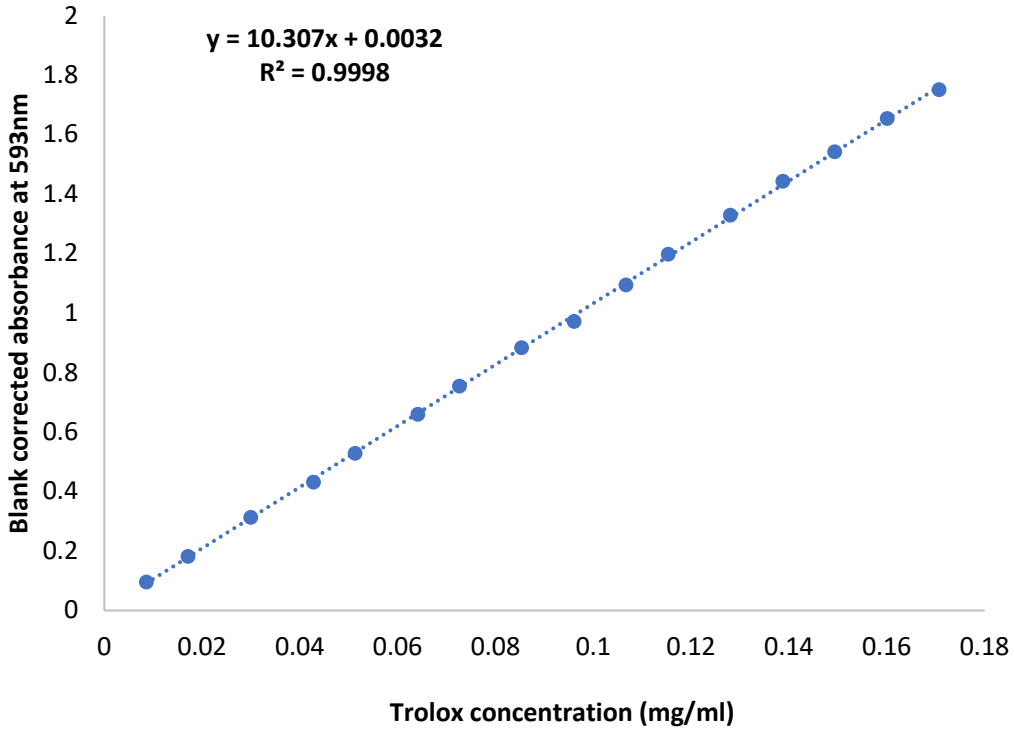
ZHU, Y., YUEN, M., LI, W., YUEN, H., WANG, M., SMITH, D. & PENG, Q. 2021. Composition analysis and antioxidant activity evaluation of a high purity oligomeric procyanidin prepared from sea buckthorn by a green method. *Current Research in Food Science,* 4**,** 840-851.

ZIELINSKI, A. A. F., HAMINIUK, C. W. I., ALBERTI, A., NOGUEIRA, A., DEMIATE, I. M. & GRANATO, D. 2014. A comparative study of the phenolic compounds and the in vitro antioxidant activity of different Brazilian teas using multivariate statistical techniques. *Food Research International,* 60**,** 246-254.

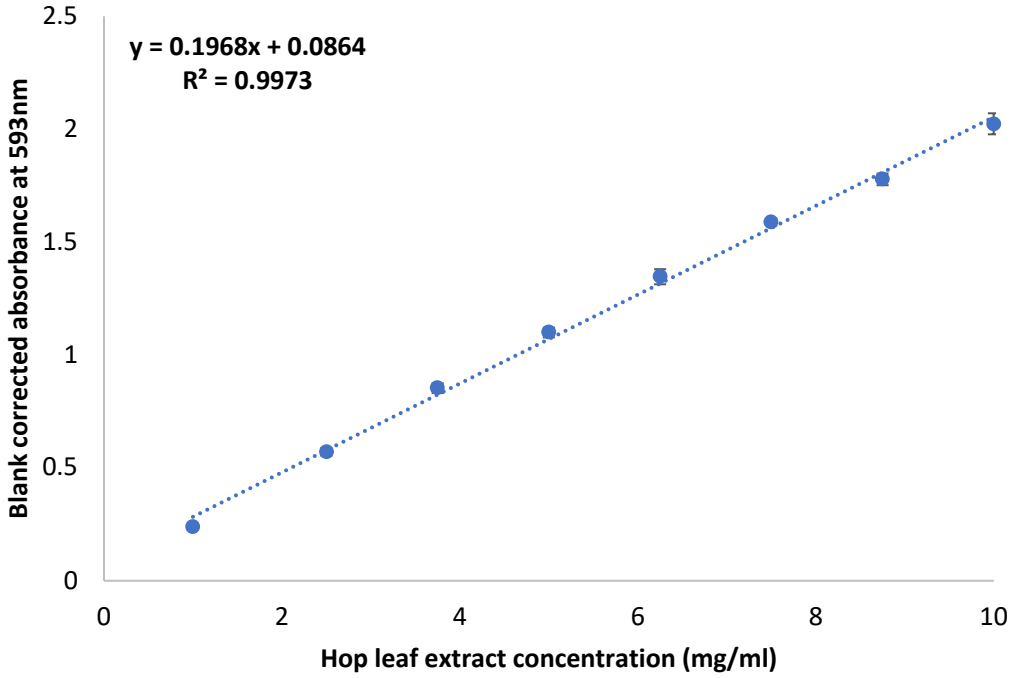
БРУСНИЦЫНА, В. 2020. The Vegan Community in Britain: Recent Trends.

Appendix

Appendix 2

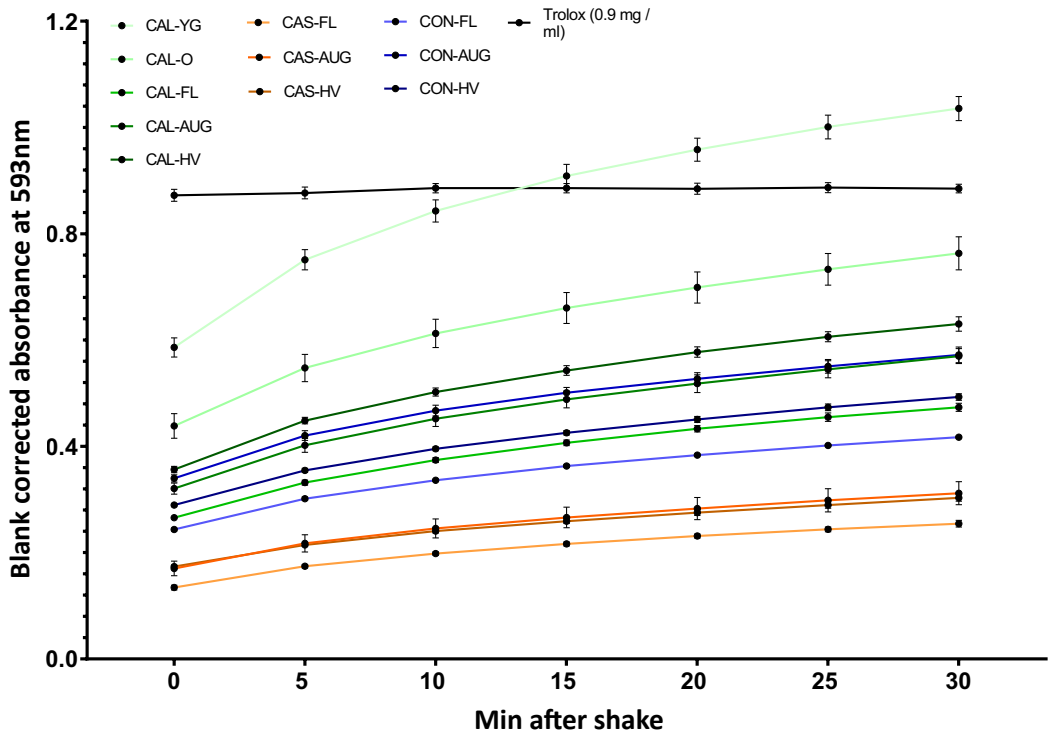


Appendix 2-Figure 1: FRAP antioxidant analysis of Trolox analysed at different concentrations between 0.01-0.18 mg/ml.



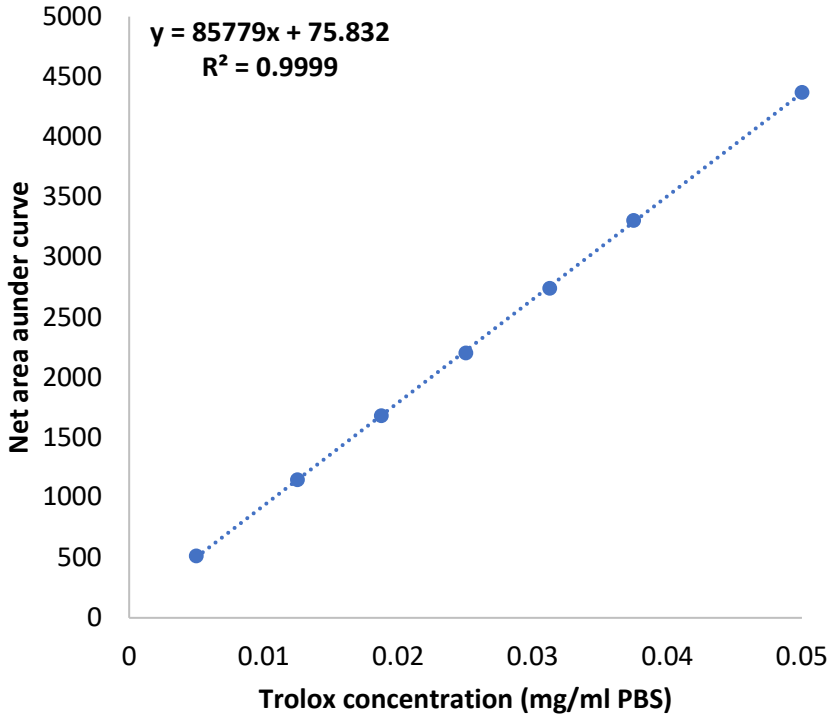
Appendix 2-Figure 2: FRAP antioxidant analysis of a hop leaf extract analysed at different concentrations between 1-10 mg/ml.

Hop leaf extract: Aqueous ethanolic (50% v/v) extract of Calypso hop leaf (2021 harvest, flower stage).



Appendix 2-Figure 3: Absorbance at 593 nm measured at 5 min intervals over 30 mins for hop leaf extracts (5 mg/ml) and Trolox (0.9 mg/ml).

CAL-Calypso. **CAS**-Cascade. **CON**-Contessa. **FL**-Flower stage, **MID**-Middle stage, **HV**-Harvest stage. **21**-2021 crop year. **22**-2022 crop year.



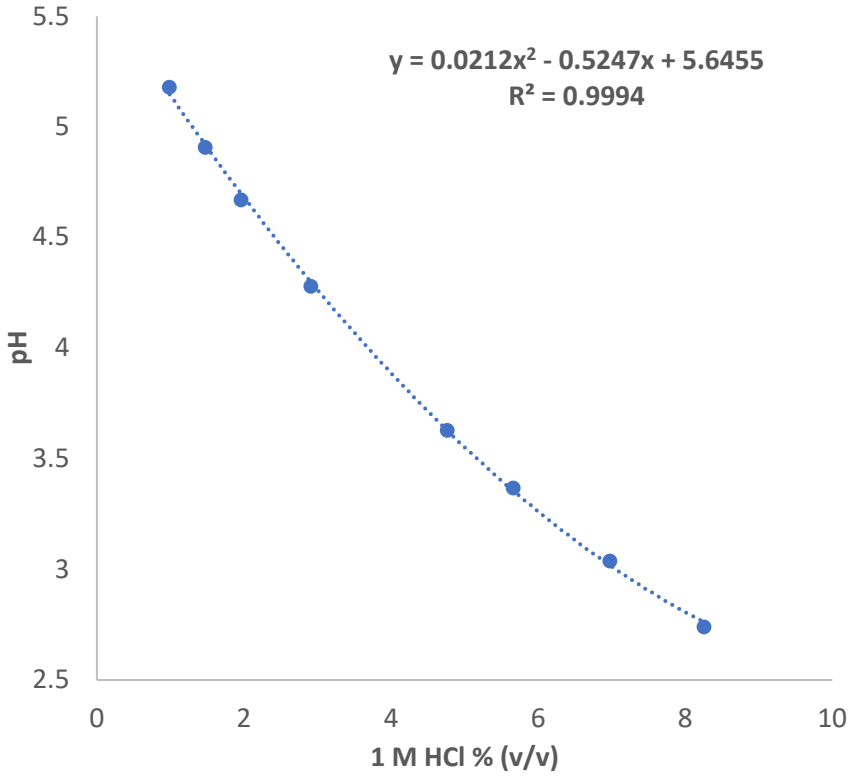
Appendix 2-Figure 4: ORAC standard curve for Trolox analysed at concentrations of 0, 0.005, 0.0125, 0.01875, 0.025, 0.03125, 0.0375 and 0.05 mg/ml in PBS.

Appendix 3

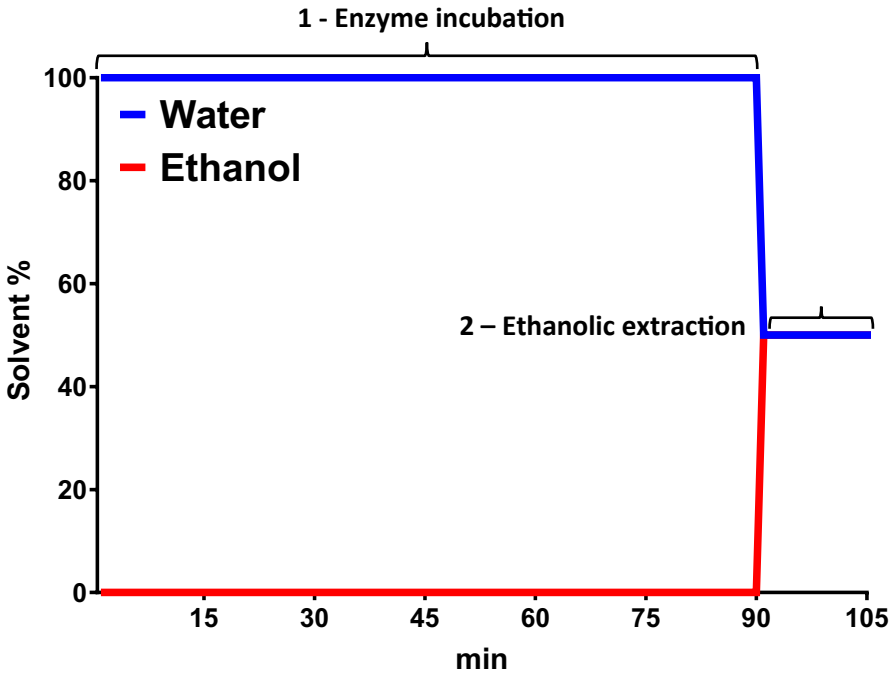
Appendix 3-Table 1: Experimental runs of the two-factor central composite design and response values to evaluate the effect of ethanol concentration (A) and solid: liquid ratio (B) on the extraction of total phenolic content (TPC), proanthocyanidin content (PAC), xanthohumol, total alpha acids and FRAP antioxidant activity.

Run	Ethanol concentration (%)	Solid: liquid ratio (mg/ml)	TPC (mg CAE/g DM)	PAC (mg PB3E/g DM)	Xanthohumol (mg/g DM)	Total alpha acids (mg/g DM)	FRAP (mg TE/g DM)
1	81.2132	107.3223	55.33	9.86	6.83	4.57	13.59
2	60	125	67.24	24.86	6.11	5.42	26.32
3	30	125	63.58	26.44	0.17	1.46	25.53
4	60	125	66.05	26.09	5.90	5.29	28.29
5	38.7868	142.6777	69.64	30.61	2.59	3.07	29.92
6	60	150	67.59	25.78	6.11	5.28	32.37
7	60	125	71.51	26.14	5.89	5.32	27.34
8	60	125	71.38	26.71	6.18	5.29	29.51
9	81.2132	142.6777	55.82	9.42	7.27	4.41	13.67
10	60	125	70.37	26.94	8.46	5.46	29.03
11	38.7868	107.3223	73.54	32.18	1.38	3.43	34.69
12	60	100	71.00	27.85	5.76	5.20	28.76
13	90	125	34.73	2.80	6.86	3.68	6.78
14	50	100	78.49	31.93	4.42	4.66	31.81
15	42.5	100	74.96	-	2.18	3.97	30.78
16	45	100	72.92	-	2.84	4.37	30.29
17	47.5	100	73.92	-	3.74	4.55	30.83

Total alpha acids: Sum of cohumulone and ad/humulone.

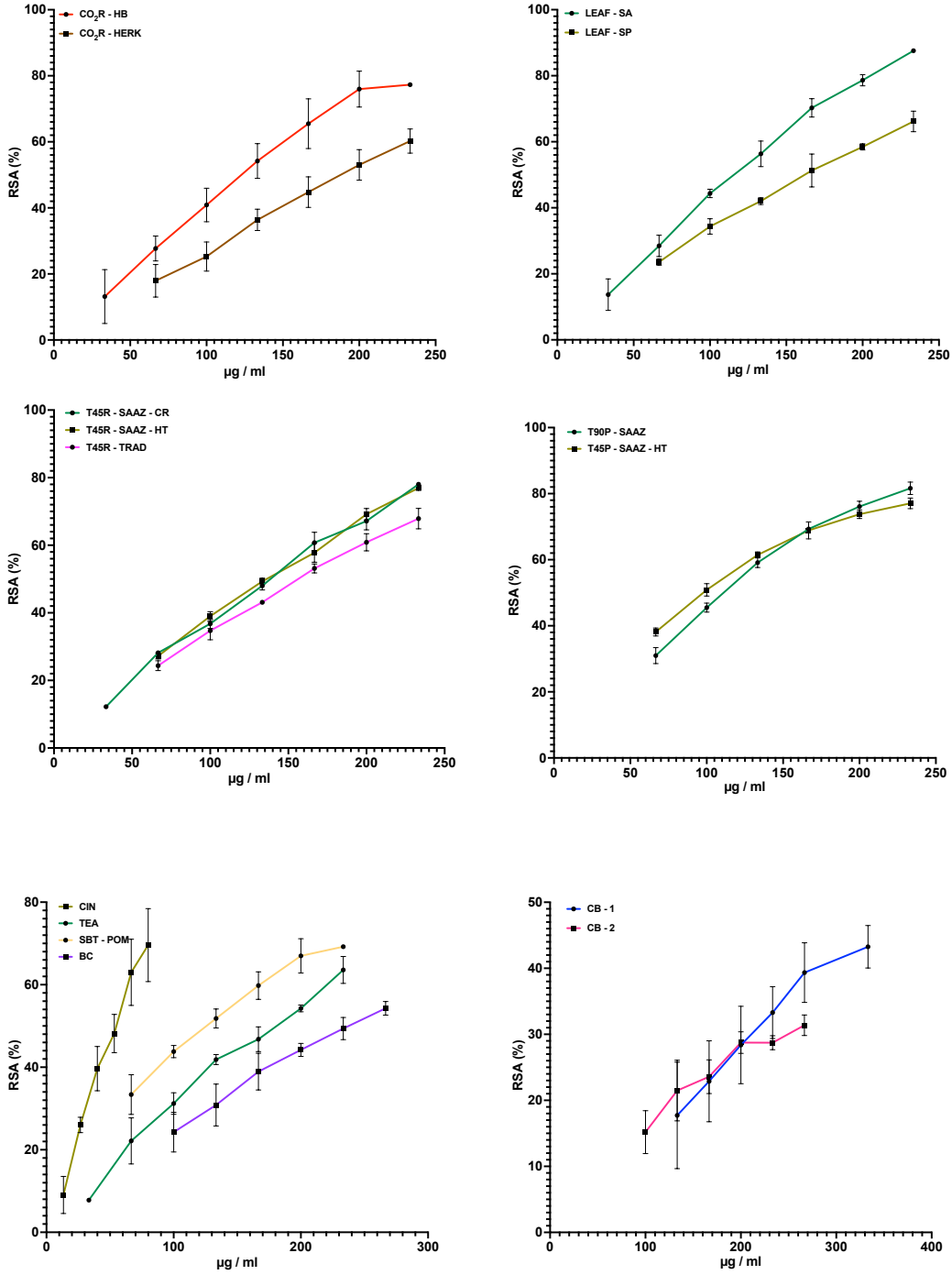


Appendix 3-Figure 1: Second order polynomial model fits used to calculate % of 1 M HCl of total solution to counteract buffering potential CO_2R-HERK extracts for enzyme assays.



Appendix 3-Figure 2: Time and solvent ratios for enzyme incubation and ethanolic extractions

Appendix 4



Appendix 4-Figure 1: DPPH RSA (%) for hop materials and comparison materials analysed between 10-350 µg/ml.

Appendix 4-Table 1: Standard curve concentrations ranges, R^2 slope and intercept values used for quantitative analysis.

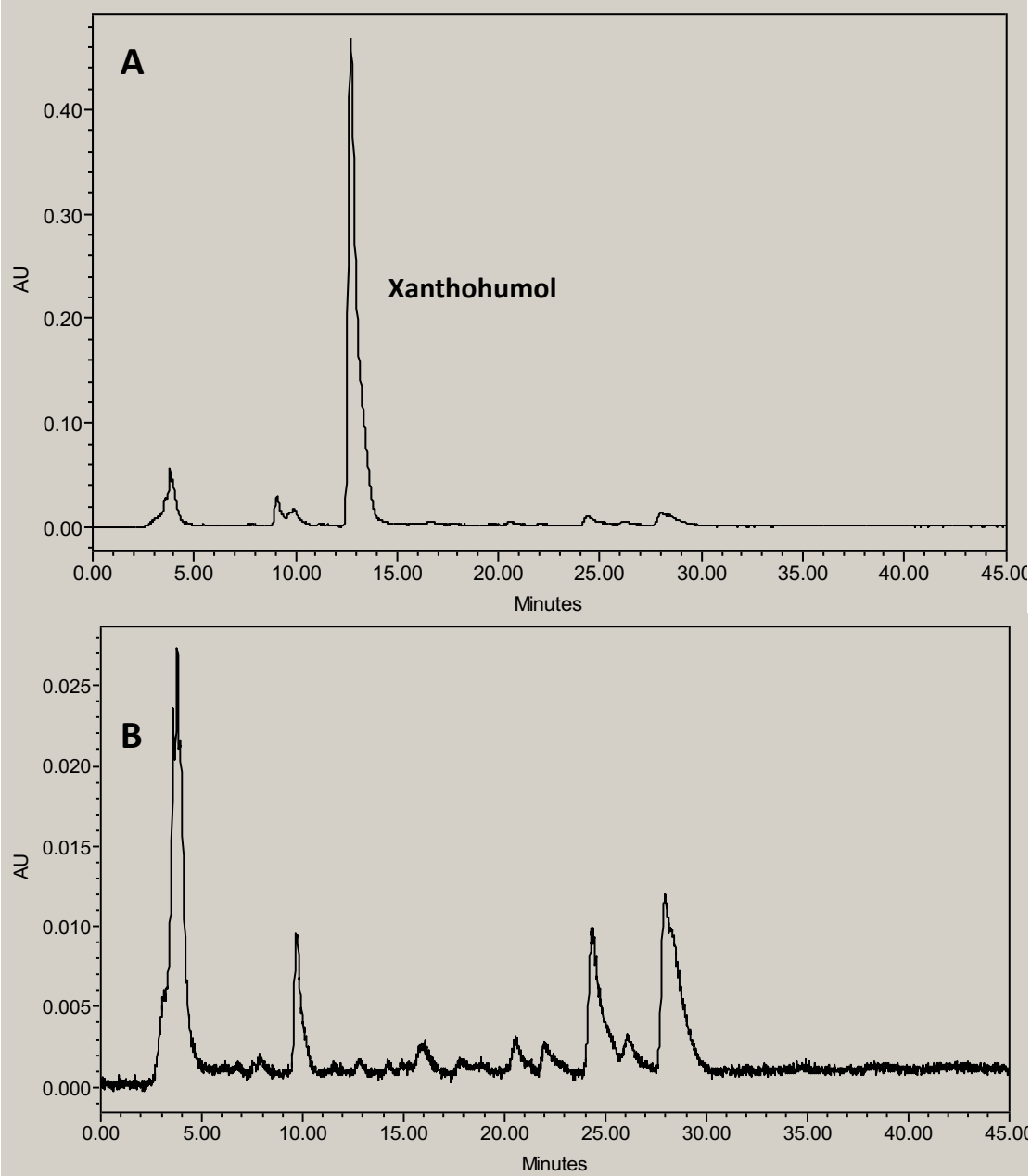
Compound	Min (µg/ml)	Max (µg/ml)	R^2	Slope	Intercept
Phenolic and chlorogenic acids					
Gallic acid	0.0019	0.3788	0.9995	0.4967	-0.0007
Protocatechuic acid	0.0019	1.8939	1	0.9582	0.0016
3,4-Dihydroxybenzoic acid	0.0038	1.8939	0.9999	0.9693	-0.0004
4-Hydroxybenzoic acid	0.0019	0.7576	0.9994	0.3948	0.0015
Caffeic acid	0.0038	7.5758	0.9992	0.2132	0.0031
Coumaric acid	0.0076	0.7576	0.9997	0.753	0.0036
Ferulic acid	0.0019	1.8939	0.9988	0.394	-0.0019
3-Hydroxybenzoic acid	0.0076	0.7576	0.9997	0.4698	0.0016
Sinapic acid	0.0019	0.3788	0.9998	0.2138	0.0013
Trans-3-hydroxycinnamic acid	0.0019	0.1894	0.999	0.7969	0.0013
Vanillic acid	0.0379	1.8939	0.9967	0.0499	0.0022
Caffeic acid	0.0038	1.8939	0.9997	0.23	-0.0005
Chlorogenic acid	0.0027	26.515	0.9993	0.2761	0.0221
3,4-Dicaffeoylquinic acid	0.0038	3.7879	0.9993	0.4816	-0.0051
Prenylflavonoids					
6-PN	0.0008	0.7576	0.9997	5.6531	0.0253
Isoxanthohumol	0.0007	2.6515	0.9997	1.2952	0.014
Xanthohumol	0.0015	3.0303	0.9998	2.5619	0.0216
Other					
Phloroglucinol	0.0379	1.8939	0.9992	0.0299	0.0001
Vanillin	0.189	0.7576	0.9979	0.0848	0.0005
Naringenin	0.0008	0.0758	0.9948	1.1732	0.0034
Hesperetin	0.0038	0.1894	0.9996	1.0974	0.0047
Flavonols and glycosides					
Kaempferol	0.0038	0.7576	0.9994	0.5794	0.0021
Quercetin	0.0038	3.7879	0.9962	0.4947	0.0024
Taxifolin	0.0004	0.0758	0.9992	0.5485	0.0009
Rutin	0.0027	26.515	0.9996	0.3671	-0.0068
Flavanols/procyanidins					
Procyanidin A1	0.0038	0.3788	0.9978	0.2434	0.0018
Procyanidin A2	0.0019	0.3788	0.9998	0.2535	0.0015
Procyanidin B1	0.0066	26.515	0.9994	0.2952	0.0217
Procyanidin B2	0.0066	26.515	0.9998	0.3189	-0.0151
Procyanidin B3	0.0066	26.515	0.9996	0.2402	0.0125
Procyanidin C1	0.0076	3.7879	0.9992	0.2194	0.0038
Catechin	0.1326	26.515	0.9997	0.1235	0.0168
Epicatechin	0.0066	6.6288	0.9997	0.467	0.0128
Epigallocatechin gallate	0.0076	0.3788	0.9959	0.3368	-0.004
Epicatechin gallate	0.0076	7.5758	0.9996	0.223	0.0004

Appendix 4-Table 2: Correlations between phenolic variables and principal components 1-3.

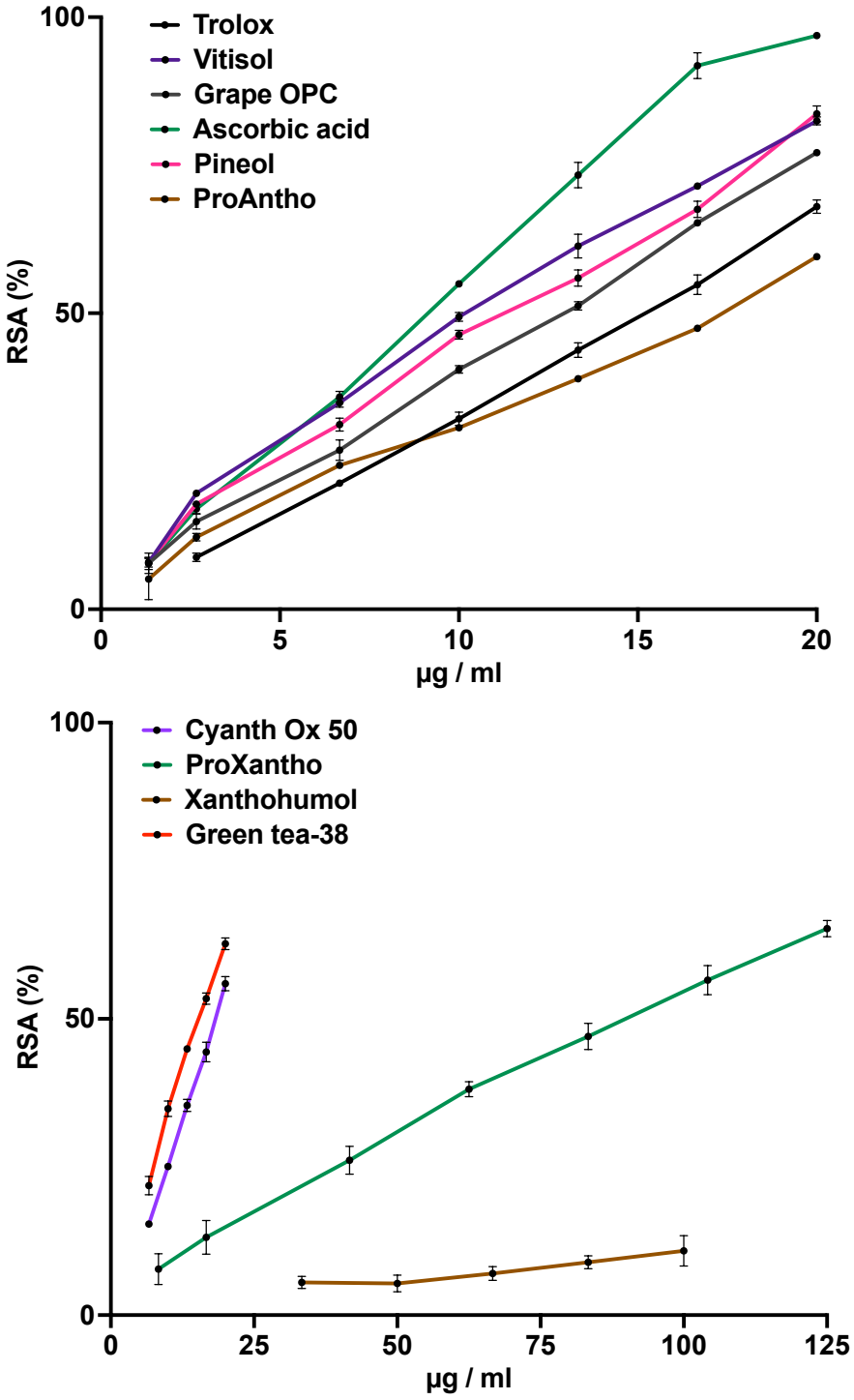
	PC1	PC2	PC3
Ascorbate (MS2)	0.104	0.170	0.221
Gallic acid (REF)	-0.674	-0.222	0.407
Protocatechuic acid (REF)	-0.432	-0.005	0.602
Gallo - Catechin B (MS2)	0.754	-0.176	0.229
4 Hydroxybenzoic acid (REF)	-0.532	0.801	0.135
Neochlorogenic acid (REF)	0.800	0.119	0.427
Salicylic acid (REF)	0.384	0.102	0.232
Gallo - Catechin A (MS2)	0.909	0.271	0.050
Catechin (REF)	-0.555	-0.615	0.401
Procyanidin B1 (REF)	-0.669	-0.272	0.628
Procyanidin B3 (REF)	-0.729	-0.284	0.566
Caffeic acid (REF)	-0.622	0.686	0.219
CQA BETA (MS2)	0.897	0.183	0.249
Chlorogenic acid (REF)	0.692	0.460	0.342
Procyanidin BD 1 (MS2)	-0.693	0.386	0.469
5-O-Feruloylquinic acid (REF)	0.774	0.124	0.218
Epicatechin (REF)	-0.357	0.599	0.638
Procyanidin B2 (REF)	-0.279	0.687	0.661
COQA G (MS2)	0.697	0.116	0.338
Coumaric acid (REF)	-0.839	0.378	-0.290
COQA D (MS2)	0.661	0.343	0.441
Procyanidin BD 2 (MS2)	-0.740	-0.362	0.525
FQA D (MS2)	0.828	-0.063	0.204
Procyanidin C1 (REF)	-0.520	0.525	0.573
Q-TG (MS2)	-0.439	0.226	-0.730
FQA B (MS2)	0.604	0.496	0.403
Taxifolin (REF)	-0.781	-0.226	0.000
Ferulic acid (REF)	-0.700	0.545	-0.160
Quercetin 3-O-neohesperidoside (MS2)	0.492	0.812	-0.083
Sinapic acid (REF)	-0.612	0.771	0.081
Quercetin 3-O-rutinoside (REF)	0.741	0.271	-0.070
Quercetin 3-O-glucoside (REF)	0.210	0.908	0.183
Quercetin 3-O-malonyl glucoside (REF)	0.513	0.185	0.791
Phloroglucinol - glycoside C (MS2)	-0.623	-0.756	0.022
Kaempferol 3-O-rutinoside (REF)	0.828	0.223	-0.060
Kaempferol 3-O-glucoside (REF)	0.683	0.590	0.335
Quercetin malonyl glucoside B (MS2)	-0.479	-0.557	0.541
4,5 Dicaffeoylquinic acid (REF)	0.602	0.678	0.283
Quercetin (REF)	-0.427	0.898	-0.043

Kaempferol (REF)	-0.215	0.937	-0.237
Naringenin (REF)	-0.743	0.562	-0.257
Hulupinic acid (MS2)	-0.516	-0.273	0.022
Isoxanthohumol (REF)	-0.612	0.527	-0.478
Desmethylxanthohumol (MS2)	-0.800	0.228	-0.093
Prenylflavonone A (MS2)	-0.583	0.734	-0.266
Curcumin (REF)	-0.623	0.421	-0.360
6-PN (REF)	-0.736	0.657	0.040
Diprenylflavonone A (MS2)	-0.489	0.852	0.001
Total Phenol Content (CAE)	-0.231	-0.076	0.541
Proanthocyanidin Content (PB3E)	-0.681	-0.332	0.565
DPPH IC50 (TE)	-0.218	-0.033	0.729
FRAP (TE)	-0.320	-0.087	0.725
ORAC (TE)	-0.615	0.383	0.286
Cohumulone (REF)	-0.610	-0.378	-0.062
Ad/Humulone (REF)	-0.607	-0.407	-0.008
Xanthohumol (REF)	-0.551	0.430	-0.583
Colupulone (REF)	-0.478	-0.673	0.172
Ad/Lupulone (REF)	-0.439	-0.773	0.220
Phenolic acid glycoside A (MS2)	-0.341	0.770	0.427

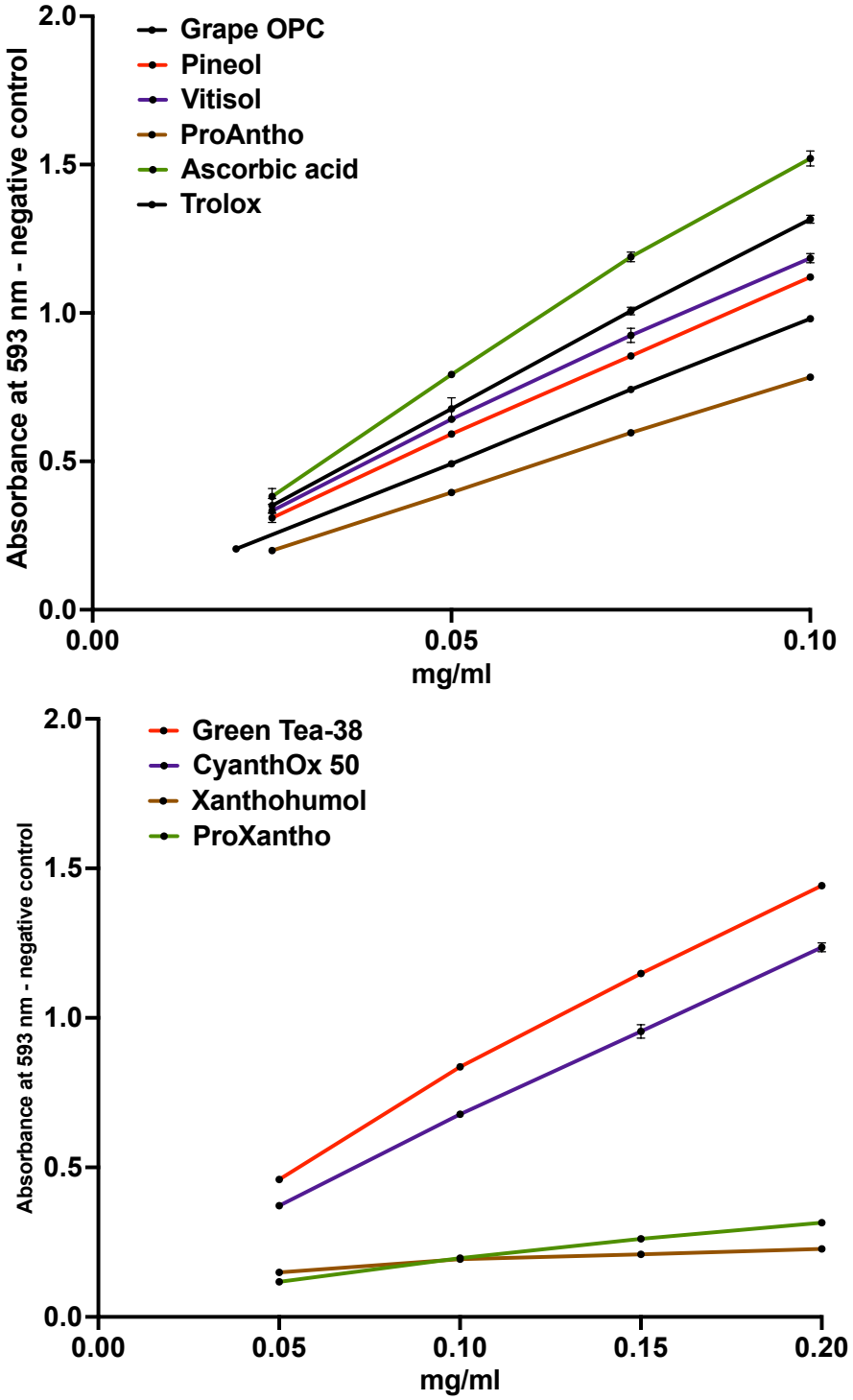
Appendix 5



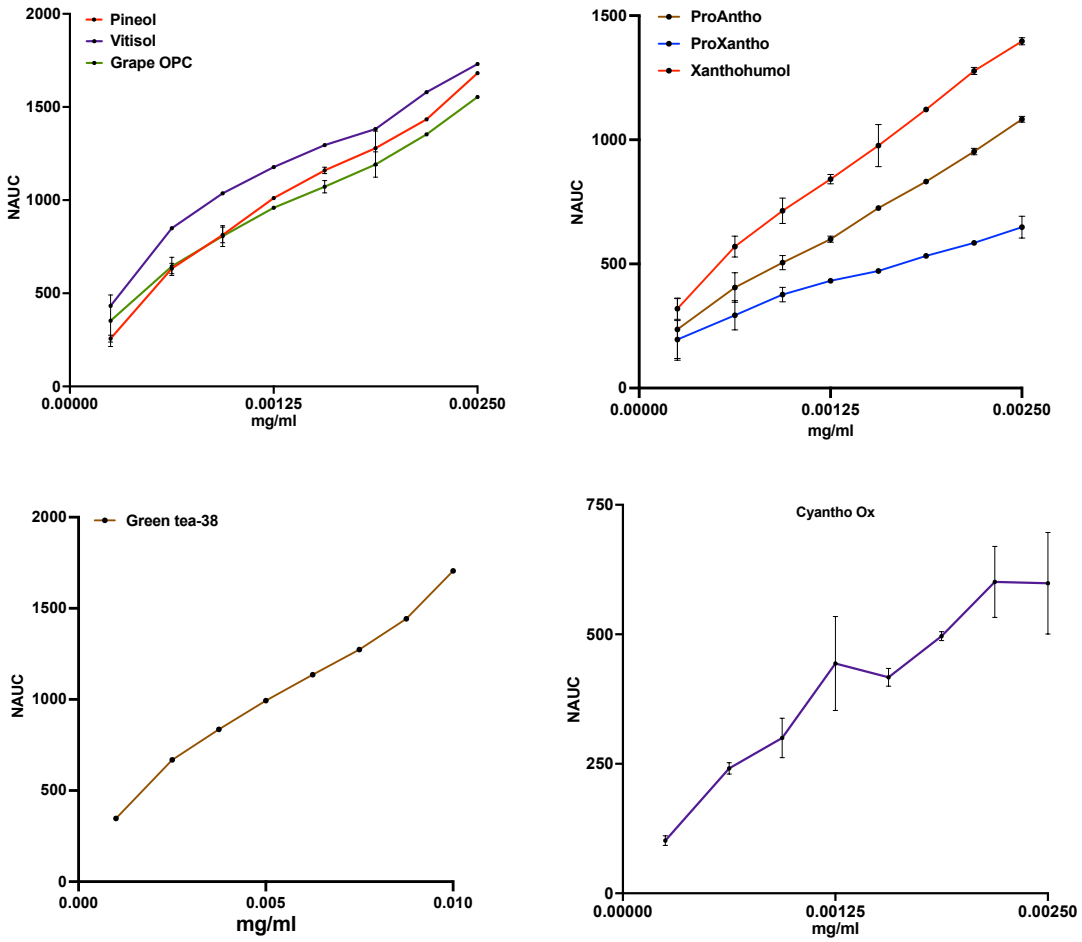
Appendix 5-Figure 1: HPLC-DAD chromatograms of CO_2R-HERK 50% aqueous extract (v/v) (A) and post PVPP adsorption solution (B) at 370 nm.



Appendix 5-Figure 2: DPPH RSA (%) for purified hop extracts, commercial extracts and phenolic standard compounds analysed at concentrations between 1.25-125 µg/ml.

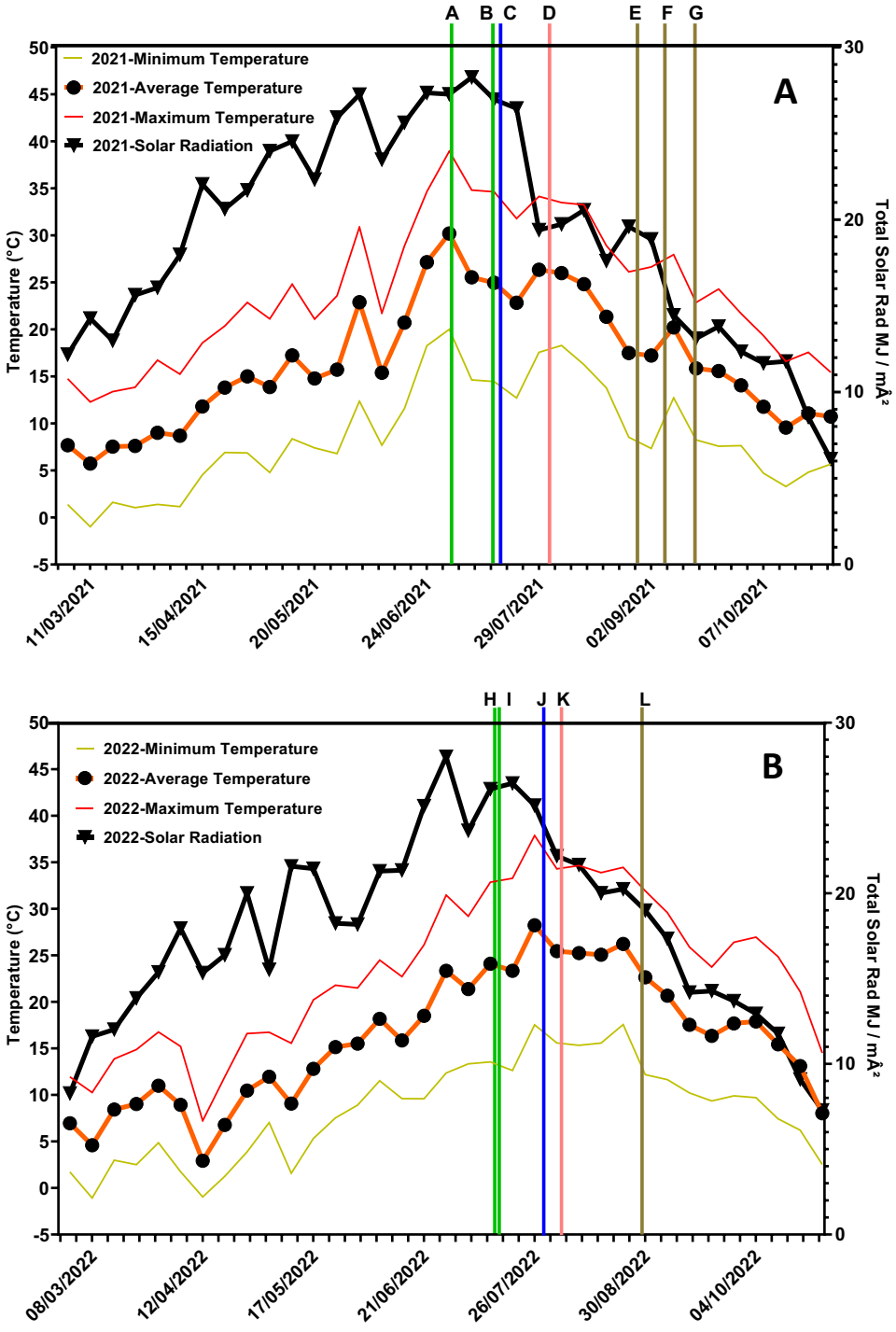


Appendix 5-Figure 3: FRAP absorbance at 593 nm - negative control of hop and commercial phenolic extracts and standard compounds analysed at multiple concentrations between 0.02-0.2 mg/ml.



Appendix 5-Figure 4: ORAC net area under curve (NAUC) of hop and commercial phenolic extracts and standard compounds analysed at multiple concentrations between 0.0005-0.010mg/ml.

Appendix 6

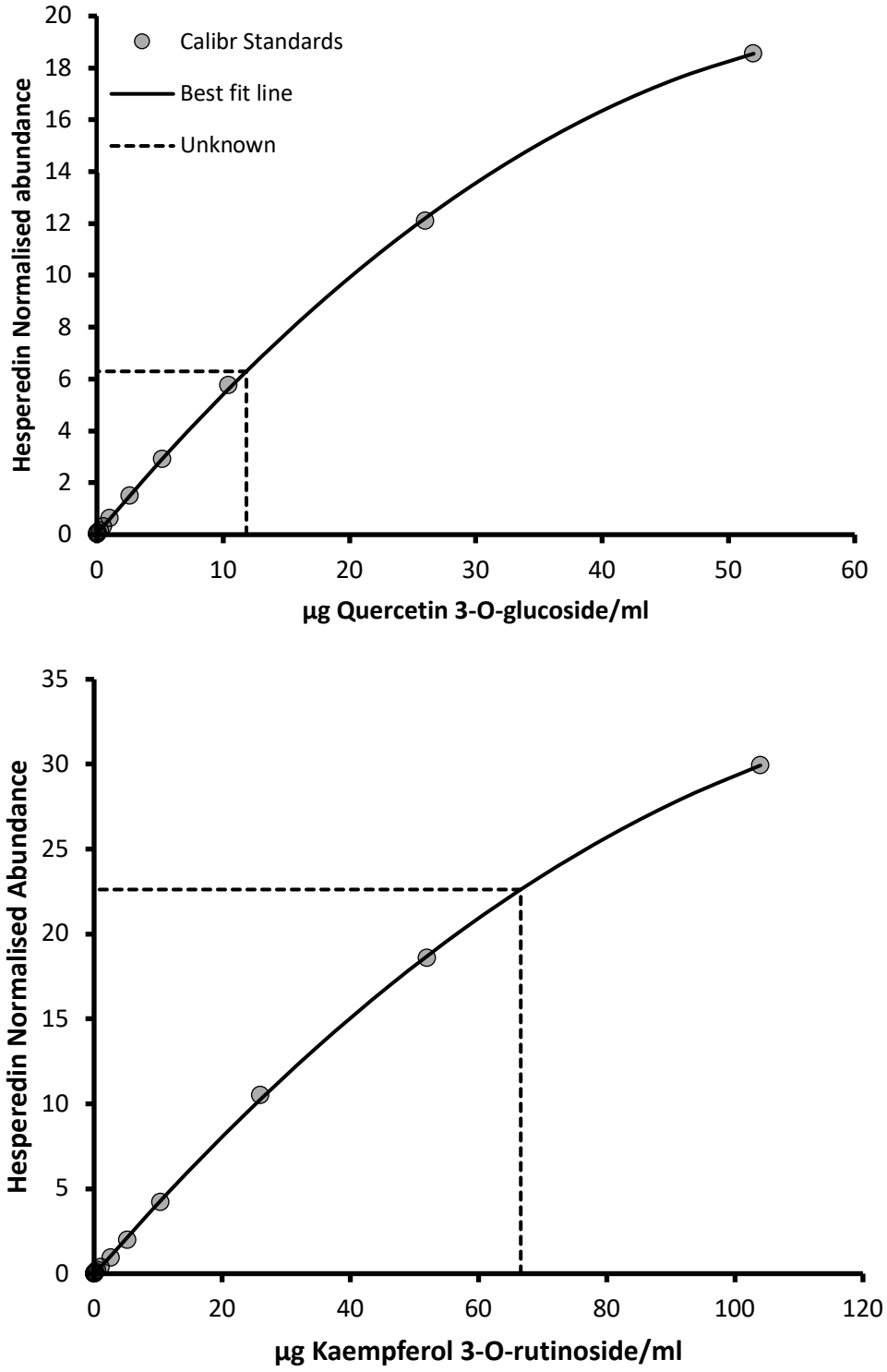


Appendix 6-Figure 1: Summary of 7-day average temperature and solar radiation data for 2021 (A) and 2022 (B). Lettering denotes the harvest dates for the leaf and cone samples for both crop years.

2021 was much hotter than 2022 especially in June/early July, peaking in the first week of July with a 7-day average temperature of 30.2 °C and highest 7-day average temperature of 38.95 °C compared to 23.33 °C and 31.48 °C respectively for 2022. There were corresponding trends for solar radiation where 2021 was consistently higher than 2022 between April and mid-July,

in particular late May/early June with a total solar radiation of 27.26 MJ/mA for 2021 and 18.18 MJ/mA for 2022. 2021 Cascade and Contessa hop plants initiated flowering earlier than in 2022 and therefore stage 1 leaves were collected earlier (A-02/07/21) than 2022 (H-13/07/22). This is likely related to elevated temperature/solar variation which can induce premature flowering as a stress response. Calypso is better suited to warmer climates and therefore stage 1 leaves were collected later for 2021 (B-13/07/21) and 2022 (I-14/07/22). These varieties have very similar photoperiod responses and typically flower together, although 2021 was an exception. For 2021 a significant outbreak of two spotted spider mites was observed possibly due to drought stress which also may have contributed to the early flowering.

Stage 4 and 5 Calypso leaves were collected mid-July for 2021 (C-15/07/21) and late July for 2022 (K-29/07/22) whilst stage 2 leaves were collected early August for 2021 (D-02/08/21) and 2022 (04/08/22). Stage 3 2021 leaves were collected on different days for Contessa (E-27/08/21), Cascade (F-06/09/21) and Calypso (G-15/09/21) whilst 2022 stage 3 leaves were all collected in late August (L-28/08/22).



Appendix 6-Figure 2: Quadratic polynomial calibration fits for quantitative analysis of kaempferol 3-O-rutinoside and quercetin 3-O-glucoside.

Appendix 6-Table 1: External and internal standards used for quantitative analysis.

Standard	Lowest Conc. (µg/ml)	Highest Conc. (µg/ml)	Internal Standard	Slope	Intercept	R^2
Protocatechuic acid	0.002	0.8	Hesperidin	0.651	0.0085	0.9997
Ferulic acid	0.004	2	Daidzein	0.3989	0.0022	0.999
Coumaric acid	0.002	0.2	Hesperidin	0.97	0.0042	0.999
Neochlorogenic acid	0.014	14	3,4-Dicaffeoylquinic acid	1.1994	-0.0346	0.9996
Chlorogenic acid	0.007	2.8	3,4-Dicaffeoylquinic acid	0.8582	0.012	0.9996
5-O-Feruloylquinic acid	0.014	2.8	Epigallo catechin	0.2482	0.0024	0.9995
4,5-Dicaffeoylquinic acid	0.002	0.04	3,4-Dicaffeoylquinic acid	2.6327	0.0039	0.9993
Catechin	0.007	14	Epigallo catechin	0.1855	0.014	0.995
Epicatechin	0.002	2	Epigallo catechin	0.3173	0.0107	0.9996
Procyanidin B1	0.008	8	Epigallo catechin	0.2113	0.0024	0.9996
Procyanidin B2	0.002	0.8	Epigallo catechin	0.1984	0.0012	0.9998
Procyanidin B3	0.002	8	Epigallo catechin	0.1585	0.008	0.9984
Procyanidin C1	0.002	0.8	Epigallo catechin	0.1346	0.0013	0.9991
Kaempferol 3-O-glucoside	0.024	4.8	Hesperidin	0.63	0.0202	0.9994
Kaempferol 3-O-rutinoside	0.026	26	Hesperidin	0.4054	-0.0237	0.9998
Kaempferol 3-O-galactoside	0.012	4.8	Hesperidin	0.4330	-0.0029	0.9996
Quercetin 3-O-malonyl glucoside	0.026	26	Hesperidin	0.1088	0.0096	0.9995
Quercetin 3-O-glucoside	0.026	26	Hesperidin	0.5527	0.0378	0.999
Quercetin 3-O-rutinoside	0.026	10.4	Hesperidin	0.6029	-0.0347	0.999
Kaempferol	0.002	0.04	Hesperidin	1.0398	0.0313	0.9992
Quercetin	0.002	0.04	Hesperidin	0.6197	0.0045	0.9982
Xanthohumol	0.002	4	Daidzein	0.6176	0.0084	0.996
6-PN	0.002	0.8	Daidzein	0.6014	0.0015	0.9998
Isoxanthohumol	0.002	0.8	Daidzein	0.5681	0.0025	0.9998

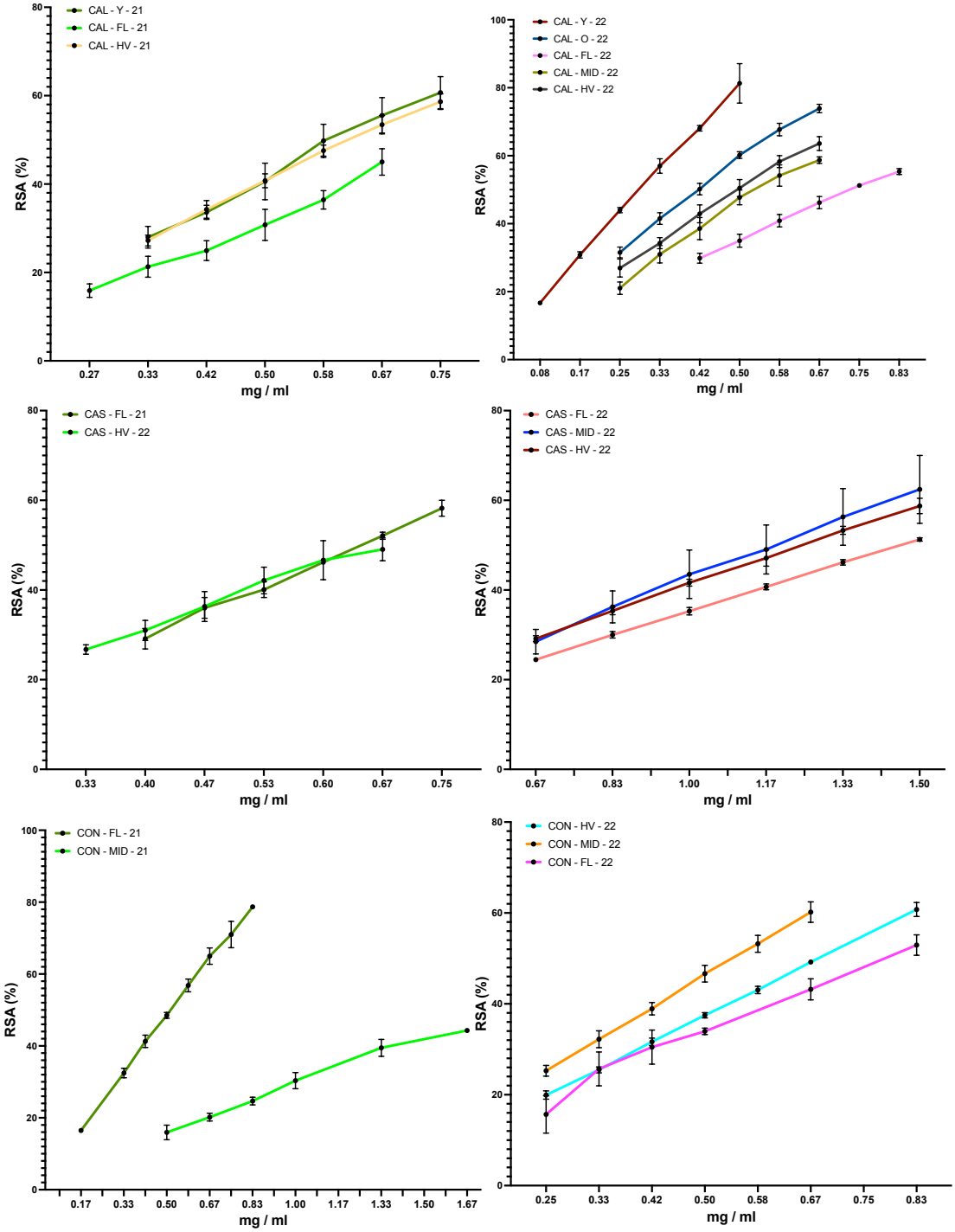
Appendix 6-Table 2: Pearson correlation coefficients for hop leaf phenolic content in relation to average temperature (Av. Temp) and solar radiation (Av. SR) for Calypso, Cascade and Contessa.

	Calypso		Cascade		Contessa	
	Av. Temp	**Av. SR**	**Av. Temp**	**Av. SR**	**Av. Temp**	**Av. SR**
Ascorbate (MS2)	0.24	**0.88**	-0.65	-0.79	-0.21	0.28
Quinate (MS2)	0.14	**0.94**	-0.19	-0.28	-0.37	-0.14
Gallo - Catechin (MS2)	0.58	-0.22	0.74	0.64	0.83	0.72
Catechin (REF)	0.84	0.55	-0.07	0.09	0.58	0.56
Epicatechin (REF)	0.85	0.25	0.42	0.41	0.76	0.68
Procyanidin B3 (REF)	0.46	0.00	0.71	0.67	0.52	0.70
Procyanidin B2 (REF)	0.45	-0.54	0.90	0.58	0.55	0.64
Procyanidin B1 (REF)	0.37	-0.31	0.89	0.42	0.31	0.49
Procyanidin B Dimer A (MS2)	0.20	0.13	0.71	0.80	0.51	0.85
Procyanidin B Dimer B (MS2)	0.39	-0.06	0.48	0.40	0.47	0.62
Procyanidin C1 (REF)	0.43	0.00	-0.43	-0.18	0.24	0.68
Neochlorogenic acid (REF)	0.29	0.97	-0.33	-0.21	0.58	0.64
Chlorogenic acid (REF)	-0.77	-0.64	-0.59	-0.35	0.61	0.74
Chlorogenic acid B (MS2)	0.47	0.92	0.42	0.41	0.83	0.61
Coumaroylquinic acid A (MS2)	0.27	**0.94**	-0.44	-0.53	0.33	0.12
Coumaroylquinic acid E (MS2)	-0.70	-0.14	-0.72	-0.46	0.70	0.61
5-O-Feruloylquinic acid (REF)	-0.17	0.63	-0.65	-0.28	-0.10	0.86
Feruloylquinic acid G (MS2)	-0.61	0.06	**-0.93**	-0.51	-0.82	0.26
Feruloylquinic acid B (MS2)	-0.03	0.58	0.82	0.77	0.80	0.52
Feruloylquinic acid D (MS2)	0.30	0.61	0.63	0.59	0.67	0.80
Coumaroylquinic acid D (MS2)	-0.71	0.04	-0.86	-0.55	0.00	0.30
Coumaroylquinic acid G (MS2)	0.07	0.54	-0.16	-0.16	0.77	-0.33
Coumaroylquinic acid B (MS2)	0.22	0.55	0.03	-0.10	0.76	-0.04
Protocatechuic acid (REF)	-0.19	0.43	-0.06	0.19	**-0.92**	0.10
Ferulic acid (REF)	-0.32	0.58	0.56	-0.06	0.74	-0.20
Coumaric acid (REF)	-0.05	0.60	-0.73	-0.69	0.22	-0.72
Phloroglucinol glycoside-A (MS2)	0.78	0.08	-0.19	-0.54	-0.20	-0.10
Phloroglucinol glycoside-C (MS2)	0.47	-0.27	**-0.89**	-0.64	0.06	0.26
4,5-Dicaffeoylquinic acid (REF)	-0.02	-0.77	0.37	-0.19	0.62	-0.53
Kaempferol 3-O-glucoside (REF)	-0.01	-0.79	0.34	-0.17	0.64	-0.48
Kaempferol 3-R-7,4-DIGALAC (MS2)	-0.32	0.55	0.85	0.60	0.13	**0.95**
Kaempferol 3,7-	-0.76	-0.84	0.10	0.09	0.58	-0.61

diglucoside (MS2)						
Kaempferol 3-O-malonyl-glucoside (MS2)	0.54	0.87	0.49	0.29	0.61	0.45
Kaempferol 3-O-rutinoside (REF)	0.57	0.55	0.79	0.42	0.78	0.10
Kaempferol 3-O-galactoside (REF)	-0.08	-0.85	-0.79	-0.52	-0.42	-0.46
Kaempferol 3-O-neohesperidoside (MS2)	0.15	-0.30	-0.64	-0.42	0.27	-0.20
Clitorin (MS2)	0.24	0.82	0.08	-0.01	0.57	0.65
Manghaslin (MS2)	0.59	0.82	-0.48	-0.31	0.62	0.59
Quercetin 3-malonyl glucoside (REF)	0.71	**0.93**	-0.64	-0.42	0.59	0.41
Quercetin malonyl glucoside B (MS2)	0.29	-0.01	-0.78	-0.51	0.32	-0.15
Quercetin 3-O-glucoside (REF)	0.08	-0.66	-0.75	-0.50	0.51	-0.29
Quercetin 3-O-rutinoside (REF)	0.58	-0.10	-0.23	-0.18	0.69	0.17
Quercetin 3-O-Neohesperidoside (MS2)	0.00	-0.53	-0.76	-0.51	0.86	0.28
Xanthohumol (REF)	0.09	0.28	-0.80	-0.77	-0.65	-0.83
6-PN (REF)	-0.46	-0.81	-0.86	-0.76	-0.10	-0.90
Isoxanthohumol (REF)	0.60	-0.33	-0.33	-0.83	-0.05	-0.61
Prenylflavanone A (MS2)	0.23	-0.13	-0.01	-0.17	-0.49	-0.01
Desmethylxanthohumol (MS2)	0.29	0.40	-0.73	-0.51	-0.21	-0.16
Hulupinic acid (MS2)	0.10	-0.49	**-0.90**	-0.87	0.14	-0.59
Cohumulone (REF)	-0.63	-0.76	-0.79	-0.52	-	-
Ad/Humulone (REF)	-0.63	-0.76	-0.79	-0.52	-	-
Colupulone (REF)	-0.52	-0.40	**-0.95**	-0.77	-0.42	-0.46
Ad/Lupulone (REF)	-0.23	-0.03	-0.89	-0.84	-0.42	-0.46
Total Phenol Content	0.48	-0.31	-0.26	-0.13	0.50	0.58
Proanthocyanidin Content	0.24	-0.39	**0.98**	0.66	0.16	0.58
Quercetin (REF)	0.50	-0.10	-0.26	-0.01	0.41	0.45

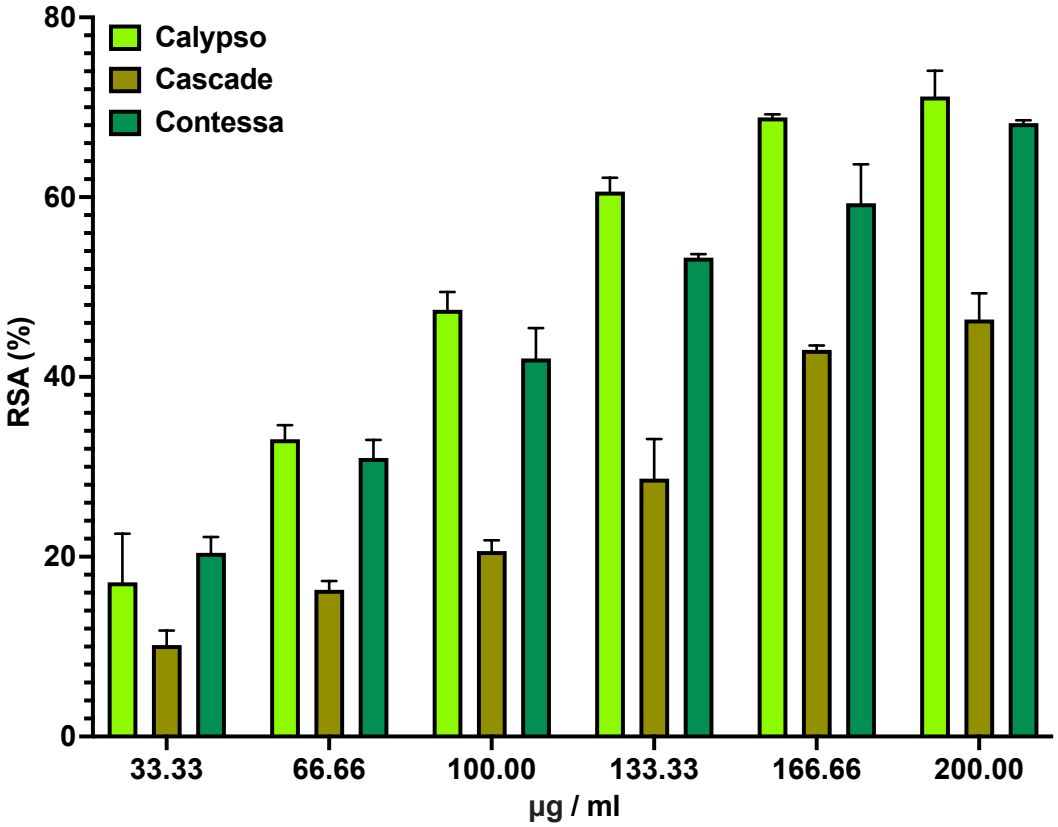
Correlations where p = <0.05 in bold.

Appendix 7

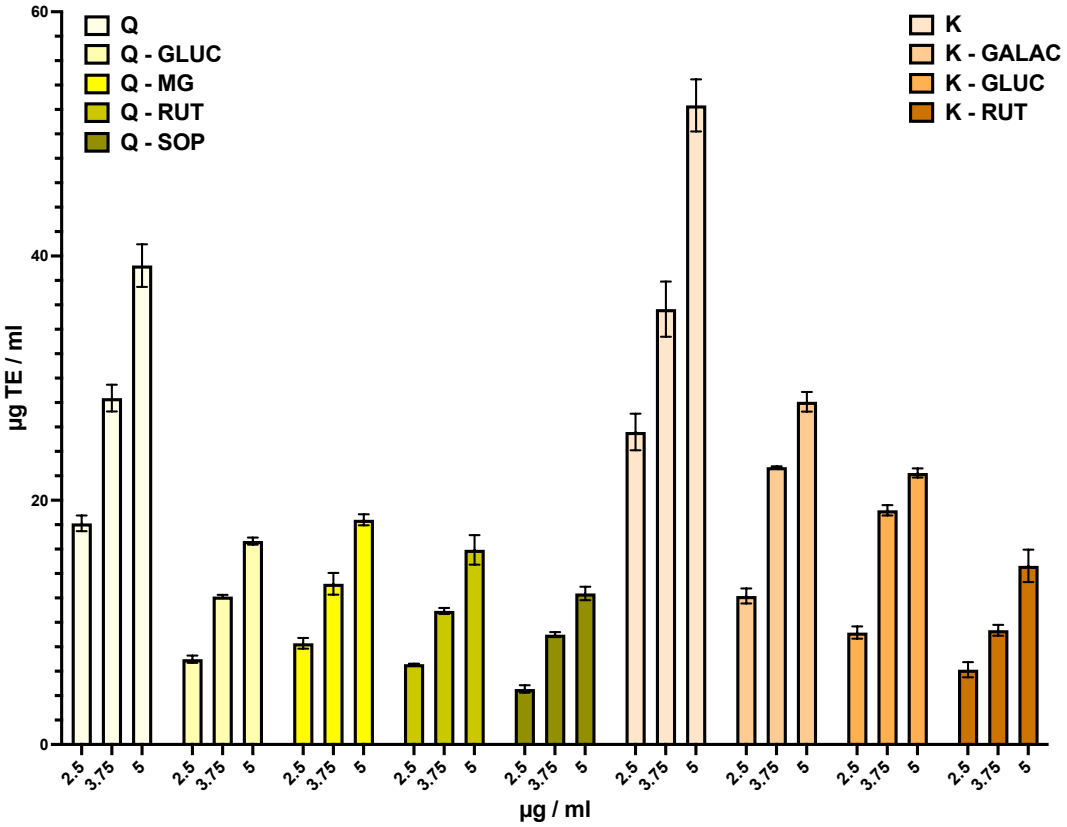


Appendix 7-Figure 1: DPPH RSA (%) for Calypso (top), Cascade (middle) and Contessa (bottom) hop leaves for 2021 (left) and 2022 (right) analysed between 0.17-1.5 mg/ml.

CAL-Calypso. **CAS**-Cascade. **CON**-Contessa. **FL**-Flower stage. **MID**-Middle stage. **HV**-Harvest stage. **Y**-Young leaves. **O**-Old leaves. **21**-2021 crop year. **22**-2022 crop year.

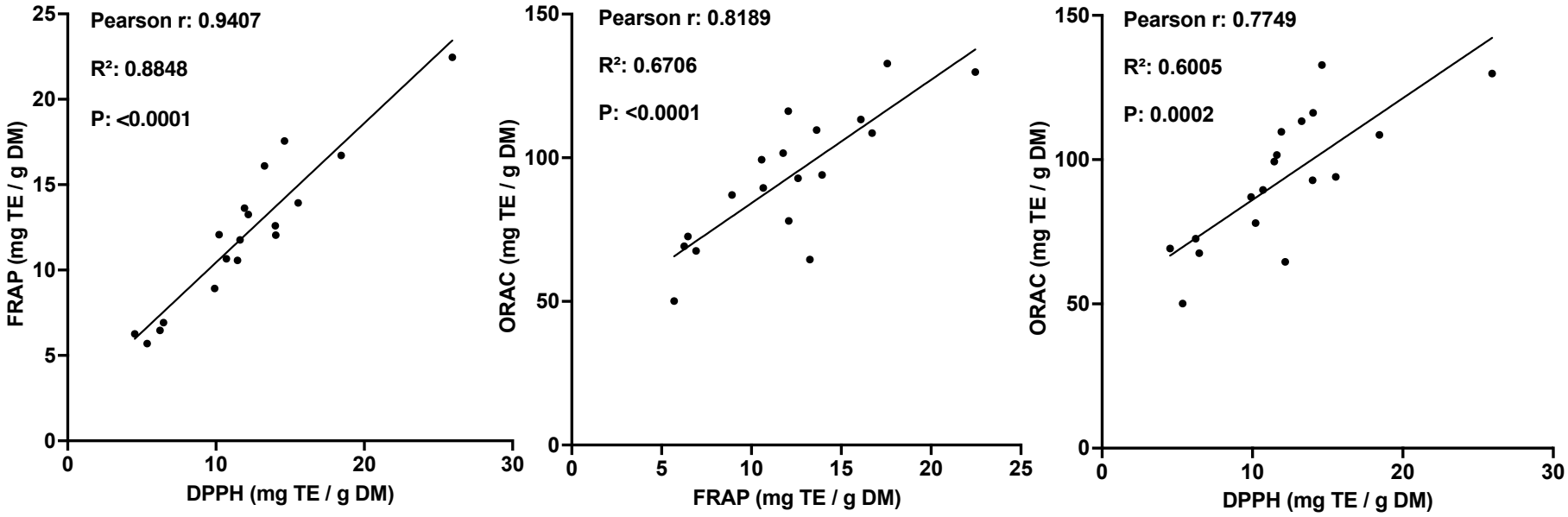


Appendix 7-Figure 2: DPPH RSA (%) for Calypso, Cascade and Contessa hop cone extracts analysed between 33.33-200 µg/ml.



Appendix 7-Figure 3: ORAC antioxidant activity of flavonol glycosides and their respective aglycone analysed at 2.5, 3.75 and 2.5 µg/ml.

TE-Trolox equivalents.



Appendix 7-Figure 4: Pearson's correlation analysis between FRAP, DPPH and ORAC antioxidant activity of hop leaf extracts (cones omitted).

TE-Trolox equivalent

Appendix 7-Table 1: Antioxidant (DPPH, FRAP and ORAC) and TPC analysis of reference phenolic compounds expressed on a weight basis as equivalents of Trolox (TE) and caffeic acid (CAE).

	mg DPPH TE/g	mg FRAP TE/g	mg ORAC TE/g	mg TPC CAE/g
		Procyanidins		
Procyanidin B2	1557.27 ± 23.23	1383.18 ± 14.88	7874.46 ± 173.19	3469.86 ± 46.19
Procyanidin B3	1146.65 ± 61.94	1362.32 ± 15.21	7233.41 ± 208.26	3140.93 ± 33.56
Procyanidin C1	1628.44 ± 15.49	1366.52 ± 9.03	7127.31 ± 125.03	3372.07 ± 53.89
		Flavonols and flavonol glycosides		
Kaempferol	511.57 ± 82.59	1935.94 ± 33.06	10467.14 ± 426.60	1433.99 ± 33.56
Kaempferol 3-O-glucoside	< LOQ	14.72 ± 1.83	5613.43 ± 161.30	< LOQ
Kaempferol 3-O-galactoside	< LOQ	10.40 ± 3.68	4446.98 ± 75.00	< LOQ
Kaempferol 3-O-rutinoside	< LOQ	< LOQ	2925.20 ± 265.58	< LOQ
Quercetin	1491.57 ± 69.68	2848.85 ± 9.18	7844.76 ± 348.73	3349.85 ± 13.33
Quercetin 3-O-glucoside	900.28 ± 7.74	668.48 ± 28.37	3331.66 ± 58.61	1180.62 ± 7.69
Quercetin 3-O-malonyl glucoside	730.56 ± 92.91	833.25 ± 4.65	3680.40 ± 91.49	1687.372 ± 46.83
Quercetin 3-O-rutinoside	624.72 ± 103.23	567.49 ± 13.35	3187.31 ± 242.25	993.93 ± 7.69
Quercetin 3-O-sophoroside	619.24 ± 12.90	546.53 ± 11.02	2473.57 ± 110.86	1038.38 ± 15.39
6-PN	< LOQ	< LOQ	6379.22 ± 151.55	393.83 ± 73.44
8-PN	< LOQ	0.58 ± 0.53	8677.72 ± 53.70	51.56 ± 20.37
Xanthohumol	< LOQ	73.29 ± 6.46	5431.57 ± 156.49	371.61 ± 7.69
Isoxanthohumol	< LOQ	23.90 ± 6.13	4257.36 ± 0.00	69.34 ± 13.33
		Chlorogenic acids		
Neochlorogenic acid	772.54 ± 12.90	908.00 ± 19.98	3598.88 ± 169.01	2300.80 ± 223.67
Chlorogenic acid	425.80 ± 18.32	695.75 ± 5.65	3086.61 ± 138.27	1874.06 ± 20.37
5-O-Feruloylquinic acid	166.65 ± 7.74	581.71 ± 7.62	2057.19 ± 81.75	753.89 ± 7.69

Appendix 7-Table 2: Antioxidant (DPPH, FRAP and ORAC) and TPC analysis of reference phenolic compounds expressed on a molar basis as equivalents of Trolox (TE) and caffeic acid (CAE).

	mM DPPH TE/g	mM FRAP TE/g	mM ORAC TE/M	mM TPC CAE/M
		Procyanidins		
Procyanidin B2	3597.64 ± 53.66	3196.96 ± 34.39	18200.30 ± 400.30	11142.26 ± 148.33
Procyanidin B3	2649.03 ± 143.10	3148.75 ± 35.15	16718.62 ± 481.35	10085.98 ± 107.76
Procyanidin C1	5639.16 ± 53.62	4732.16 ± 31.26	24681.33 ± 432.96	16223.44 ± 259.29
		Flavonols and flavonol glycosides		
Kaempferol	585.00 ± 94.44	2213.84 ± 37.80	11969.68 ± 487.84	2278.273 ± 53.31
Kaempferol 3-O-glucoside	< LOQ	26.37 ± 3.28	7966.54 ± 134.36	< LOQ
Kaempferol 3-O-galactoside	< LOQ	18.63 ± 6.59	10056.19 ± 288.97	< LOQ
Kaempferol 3-O-rutinoside	< LOQ	< LOQ	6948.03 ± 630.81	< LOQ
Quercetin	1801.06 ± 84.14	3439.98 ± 11.09	9472.51 ± 421.09	5619.70 ± 22.37
Quercetin 3-O-glucoside	1669.27 ± 14.36	1240.23 ± 52.63	6181.20 ± 108.74	3041.31 ± 19.83
Quercetin 3-O-malonyl glucoside	1606.48 ± 204.31	1832.27 ± 10.23	8093.06 ± 201.17	5155.02 ± 143.07
Quercetin 3-O-rutinoside	1523.78 ± 251.81	1394.36 ± 32.79	7774.35 ± 590.87	3368.20 ± 26.09
Quercetin 3-O-sophoroside	1550.01 ± 32.30	1367.95 ± 27.58	6191.34 ± 277.48	3611.05 ± 53.54
6-PN	< LOQ	< LOQ	8675.53 ± 206.11	744.13 ± 138.77
8-PN	< LOQ	0.79 ± 0.72	11801.43 ± 73.03	97.42 ± 38.48
Xanthohumol	< LOQ	103.76 ± 9.15	7690.34 ± 221.57	730.99 ± 15.14
Isoxanthohumol	< LOQ	33.83 ± 8.68	6027.83 ± 0.00	136.40 ± 26.23
		Chlorogenic acids		

Neochlorogenic acid	1093.56 ± 18.27	1285.32 ± 28.28	5094.36 ± 239.24	4524.84 ± 439.88
Chlorogenic acid	602.73 ± 259.39	984.86 ± 8.00	4369.23 ± 195.72	3685.61 ± 40.06
5-O-Feruloylquinic acid	245.24 ± 11.39	856.01 ± 11.21	3027.26 ± 120.30	1541.32 ± 15.74

Appendix 7-Table 3: Concentrations of xanthohumol, catechin and epicatechin in snailase treated and control extracts (mg/kg).

	Cascade		**S. Passion**		**S. Aroma**	
	Control	**Snailase**	**Control**	**Snailase**	**Control**	**Snailase**
Xanthohumol	1316.05 ± 67.82 **A**	1422.90 ± 103.63 **A**	379.80 ± 30.94 **B**	391.36 ± 46.23 **B**	274.83 ± 42.98 **B**	332.02 ± 40.89 **B**
Catechin	205.89 ± 16.33 **B**	232.06 ± 47.53 **B**	322.14 ± 67.79 **B**	360.39 ± 30.75 **B**	1916.12 ± 30.57 **A**	1893.20 ± 124.33 **A**
Epicatechin	126.31 ± 29.04 **B**	196.23 ± 55.12 **B**	248.41 ± 41.11 **B**	348.25 ± 45.68 **B**	1433.09 ± 33.35 **A**	1427.77 ± 103.12 **A**

Letters represent ANOVA post-hoc groupings ($P<0.05$).