Reducing power of various hop varieties

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ABSTRACT

Since lipid autoxidation during wort boiling is determinant for the appearance of staling flavour in aged beers, the reducing power of hops added in the boiling kettle was investigated. An assay based on the inhibition of linoleic acid oxidation in the presence of an initiator (2,2'-azobis(2-amidino-propane) dihydrochloride = AAPH) enabled us to distinguish hop varieties and conditionings. Large differences in hop flavanoid contents explained the higher antioxidant activity of low-α-acid samples versus bitter varieties and CO_2 hop extracts. When the α-acids reducing power was subtracted, very good correlation was observed between the resulting inhibition time and the amount of total flavanoids in pellets. An analysis of the hop polyphenols content explains this result since flavanoids represent more than 80% of phenolic compounds. As expected, adding hop pellets to the kettle effectively increased the overall reducing activity of wort. Supercritical CO_2 hop extracts had no significant effect due to their extremely low level of polyphenols. The concentration of the very well-known marker of beer ageing, *trans*-2-nonenal, was lower in wort boiled with hop exhibiting a better reducing power.

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INTRODUCTION

Flavour changes in packaged beer constitute one of the most serious problems in brewing. Among the carbonyls responsible of these changes, *trans*-2-nonenal is very important, imparting to the product a cardboard off-flavour at a concentration as low as 0.035 ppb (Meilgaard, 1993). Despite the high reducing activity of yeast, oxidation during germination, mashing and boiling appears as the key step in development of this stale flavour during beer storage (Lermusieau *et al.*, 1999). According to Collin *et al.* (1997), Noël *et al.* (1999a) and Noël *et al.* (1999b), the nonenal potential found in the wort before fermentation is derived from linoleic acid oxidation at the end of the boiling stage and from protein adducts created during kilning and mashing but dissolved during wort filtration and boiling. In this nonenal potential, *trans*-2-nonenal is protected from yeast activity by binding to nitrogenous compounds such as amino acids and proteins. *trans*-2-Nonenal would be further released by acidic hydrolysis, mainly when the beer pH is low or the storage temperature inadequate.

Most efforts in breweries now focus on inhibiting lipid autoxidation reactions in the brewhouse. Adding sulphites to the kettle proves an interesting way to limit the cardboard flavour in aged beers when too highly oxidized green worts are used (Lermusieau *et al.*, 1999). Optimising the natural reducing substances in raw materials seems, however, more attractive. A new assay recently developed by Liégeois *et al.* (1999, 2000) for assessing the relative antioxidant properties of raw materials has evidenced the exceptional intrinsic reducing activity of hops (30 times more efficient than malt).

Various hop cultivars and conditionings were thus investigated. We have compared how effectively six hop varieties (harvest 1998, T90 pellets) and two supercritical CO_2 hop extracts inhibit linoleic acid oxidation in an aqueous dispersion, relating the results to the α-acid and polyphenol contents. The reducing power was also assessed for six worts boiled in presence of different hop varieties and conditionings. Nonenal potential measurements on boiled wort (defined by Drost *et al.* (1990) as the potential of wort to release *trans*-2-nonenal when cooked 2 hours at 100°C and pH 4 under Argon), were used to assess the wort reducing power influence on the nonenal synthesis during this step (Lermusieau *et al.*, 2000).

THE AAPH REDUCING POWER TEST APPLIED TO HOPS

Natural reducing substances occurring in wort are mainly polyphenols, extracted from both malt and hops, and the products of Maillard reactions (reductones and melanoidins) (Pflugfelder, 1992). The reducing power of wort is often determined by colorimetric or electrochemical assays. As previously reported (Liégeois *et al.*, 2000), our laboratory is currently working with a new, reliable method based on linoleic acid oxidation in an aqueous solution. This oxidation is induced by the free radical initiator AAPH, or 2,2'-azobis (2-amidinopropane) dihydrochloride, represented in Figure 1 by the symbol A-N=N-A.

Azo radical initiators generate free radicals by their spontaneous thermal decomposition. They can also initiate lipid peroxidation, even at 37°C. As the lipid chosen is linoleic acid, the products resulting from peroxidation are conjugated diene hydroxyperoxides absorbing at 234 nm. It is thus easy to monitor the concentration of conjugated dienes during the oxidation assay (Liégeois *et al.*, 2000).

This method allows the analysis of aqueous solutions like wort and beer. It is also particularly appropriate for studying the intrinsic reducing power of raw materials (Liégeois *et al.*, 2000). Applied to hops, the method proves very fast and reproducible. Hop pellets are crushed in a mortar. One gram of ground sample is extracted four times with methanol (McMurrough & Madigan, 1996; Stevens *et al.*, 1999) by shaking for 15 minutes and sonicating for 5 minutes. After centrifugation, the supernatant is collected and appropriately diluted with pure methanol. As shown in Figure 2, a linear relationship is obtained between hop concentration and inhibition time. The concentration of 13.3 ppm hop in the assay was chosen for further experiments.

Initiation

$$A\text{-}N{=}N\text{-}A \longrightarrow A\text{-}A + A^{\cdot} + N_2$$

$$A^{\cdot} + O_2 \longrightarrow AO_2^{\cdot}$$

$$AO_2^{\cdot} + LH \longrightarrow AOOH + L^{\cdot}$$

Propagation

$$L^{\cdot} + O_2 \longrightarrow LO_2^{\cdot}$$

$$LO_2^{\cdot} + LH \longrightarrow L^{\cdot} + LOOH \quad \Rightarrow 234\ nm$$

Termination

$$2\ LO_2^{\cdot} \longrightarrow \text{non radical products}$$

with A =

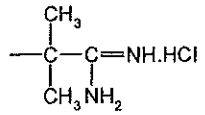


Figure 1: Principle of the AAPH oxidation assay (Liégeois *et al.*, 2000).

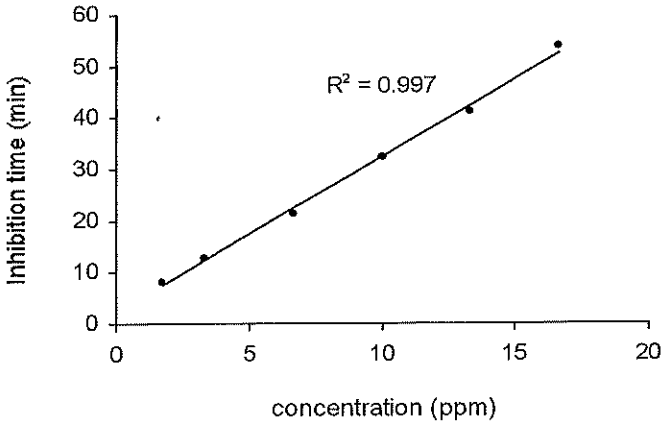


Figure 2: Relationship between the inhibition time (T_{inh}) and the hop concentration in the assay.

Four extractions and four analyses were performed on hop pellets. As displayed in Table 1, the reproducibility proved very good with a variation coefficient close to 2%. The results were similar for wort. Although linoleic acid is the major fatty acid in hop, its concentration is negligible when the sample is diluted several times.

Table 1: Reproducibility of the AAPH assay when applied to hop and wort.

Hop reducing power		Wort reducing power	
assay	T_{inh} (min)	assay	T_{inh} (min)
A	50.1	A	33.6
B	51.0	B	34.8
C	51.3	C	32.8
D	49.1	D	34.2
average = 50.4	C.V. = 1.7	average = 33.8	C.V. = 2.2

As compared with malt or other natural antioxidants, hop pellets display a very high intrinsic reducing power. The reducing efficiency of Saaz pellets, for instance, is 40 times higher than that of malt and only 4 times lower than that of ascorbic acid. As shown in Table 2, our method confirms the exceptional antioxidant power of phenolic compounds like catechin or caffeic acid.

Table 2: Relative reducing power of various antioxidants as compared with Saaz pellets.

	Relative reducing efficiency
malt	0.023
Saaz pellets	1
vitamin C	4.2
vitamin E	10.4
catechin	29.4
caffeic acid	40.1

Pellets of the Saaz, Hallertau, Tettnang, Styrian goldings, Challenger and Nugget varieties were analysed by the AAPH reducing power test. Surprisingly, very great differences appeared between hop varieties. The Saaz variety, recognized as the best one for flavour, was here characterized by the highest reducing power: 62.9 minutes as opposed to 38 for the α-rich Nugget sample (Table 3) (Lermusieau *et al.*, 2000).

Table 3: Comparison of reducing power in different varieties.

Variety		T_{inh} (*min*) 13.3 ppm hop
Saaz	(2.9% α)	62.9
Hallertau	(3.3% α)	55.4
Tettnang	(4.8% α)	52.8
Styrian Goldings	(4.4% α)	42.9
Challenger	(6.3% α)	40.3
Nugget	(11.8% α)	38.0

As already mentioned, part of the *trans*-2-nonenal appearing during beer ageing comes from linoleic acid autoxidation during the boiling step. We thus investigated the influence of hop on wort reducing power.

Table 4: Filtered wort and pitching wort reducing power measured when different hop varieties and conditionings are used at the beginning or 7 minutes before the end of the boiling step.

Boiling	T_{inh} *(min)*	T_{inh} increase *(%)*
Wort before boiling	39.3	-
Boiling without hop	36.8	0
hop at the beginning of the boiling step		
Saaz pellets (1.8 g/L)	50.2	+36
Challenger pellets (1.8 g/L)	47.4	+29
CO_2 Saaz extract (0.85 g/L)	38.2	+4
hop 7 minutes before the end of the boiling step		
Saaz pellets (1.8 g/L)	49.7	+35
Challenger pellets (1.8 g/L)	48.8	+33
CO_2 Saaz extract (0.85 g/L)	39.0	+6

As depicted in Table 4, unboiled wort has a higher reducing power than wort boiled without hop. During boiling, expectedly, part of the polyphenols derived from the malt precipitate. Reducing compounds derived from Maillard reactions (melanoidins and reductones) (Pflugfelder, 1992) cannot balance this loss in the absence of hop.

Hop contributes significant reducing power to the wort despite the low amounts used. When Saaz hop pellets are added either at the beginning or at the end of the boiling step, the pitching wort reducing power increases by 36%. As foreseeable, Challenger pellets are a bit less efficient in both experiments: the increase is only 29 to 33%. CO_2 hop extracts have almost no effect (Table 4) (Lermusieau *et al.*, 2000).

When the nonenal potential was measured in pitching worts, polyphenol-rich pellets appeared to improve organoleptic stability of the beer (Table 5). Even though we added 5 times more α-acids to the wort when the CO_2 Saaz extract was used, we measured in this case a very low inhibition time associated with a higher nonenal potential. The higher the reducing power brought to the wort by hop, the lower the amount of *trans*-2-nonenal linked to nitrogenous compounds. Hence, in agreement with the feeling of most brewers, CO_2 hop extracts emerge as the least effective at preventing staling

Table 5: Nonenal potential measurements applied to wort boiled without hop, with hop pellets and with hop CO_2 extracts.

Boiling	T_{inh} increase resulting from hop addition *(%)*	nonenal potential *(ppb)*
BLANK = no hop	-	3.2
Saaz pellets	+36	2.5
CO_2 Saaz extract	+4	3.0

As shown in Table 6, differences between varieties and processings are even more significant if the hop inhibition time is adjusted according to the α-acid content of each sample. In this case, the Saaz sample shows an eight-times-higher inhibition time than the Nugget sample. Surprisingly, the higher the α-acid concentration in hop pellets, the lower the inhibition time. This again indicates that α-acids do not contribute significantly to antioxidant activity (Lermusieau *et al.*, 2000). For CO_2 extracts, the inhibition time is always very low, whatever the cultivar. Saaz nevertheless again appears as the best one.

Table 6: Reducing power of hops, taking into account the α-acid level.

Variety		T_{inh} *(min)* 1 ppm α-acid
pellets		
Saaz	(2.9% α)	162.8
Hallertau	(3.3% α)	126.0
Tettnang	(4.8% α)	82.6
Styrian Goldings	(4.4% α)	73.2
Challenger	(6.3% α)	48.0
Nugget	(11.8% α)	24.2
CO_2 extracts		
Saaz	(28% α)	13.2
Target	(46% α)	8.7

The inhibition time measured for 1 ppm pure α-acid in the reducing power assay was 4.8 minutes. This value is very low and explains the results obtained previously with supercritical CO_2 extracts.

As some authors have evidenced an inverse correlations between α-acid and polyphenol contents in hop (Erdal *et al.*, 1985; Asano *et al.*, 1981), we logically suspected that this might partially explain our experimental reducing power values. Moreover, phenolic compounds emerge from many works as the most efficient natural antioxidants (Maillard & Berset, 1995; Saint-Cricq de Gaulejac *et al.*, 1999; Sawa *et al.*, 1999; Liégeois *et al.*, 2000).

To identify the hop reducing agents, we subtracted from all inhibition times the α-acids contribution and compared the new values with the polyphenol levels in the different hops (Figure 3). The higher the polyphenol or flavanoid content of the hop pellets (Delcour & Janssens de Varebeke, 1985; Bishop, 1972), the greater the inhibition time. These nice correlations confirm the key role of flavanoids on hop reducing power (Lermusieau *et al.*, 2000).

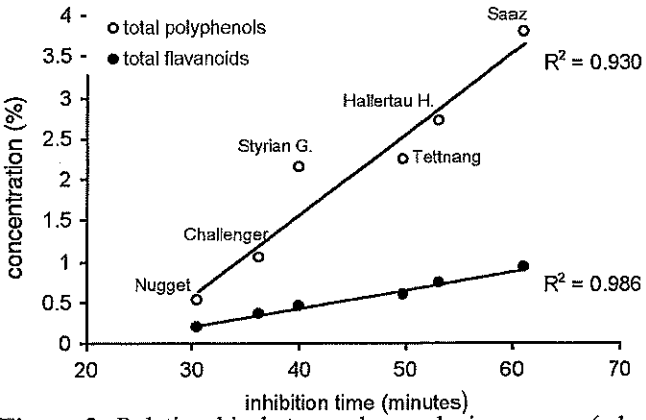


Figure 3: Relationship between hop reducing power (when the α-acid contribution is subtracted) and hop polyphenol or flavanoid contents (total flavanoids and total polyphenols are given in catechin equivalents).

HOP POLYPHENOLS

What, then, are the main polyphenols in hop? Hydroxybenzoic (< 0.01%) and hydroxycinnamic acids (0.01 – 0.03%) are present at very low concentrations (McMurrough *et al.*, 1984). The most famous polyphenols in hops are polycyclic structures called flavonoids. They can represent from 2 to 5% of hop weight, depending on the variety (Asano *et al.*, 1981; Vancraenenbroek *et al.*, 1983; McMurrough et Hennigan, 1984; De Keukeleire *et al.*, 1999). About 70-80 percent of the polyphenols in wort are derived from malt and only 20-30 percent from hops (De Keukeleire *et al.*, 1999).

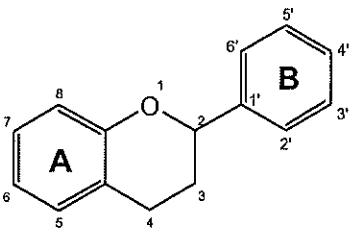


Figure 4: Flavonoid and anthocyanidin structure.

All flavonoids and anthocyanidins have a C_6-C_3-C_6 structure (Figure 4) (Rice-Evans *et al.*, 1996). Such C_{15} polyphenols are usually subdivided into four subgroups: chalcones, flavanoids, flavonols, and anthocyanidins. These last, which are coloured, charge compouds, are not found in hops and will not be discussed here (McMurrough & Hennigan, 1984).

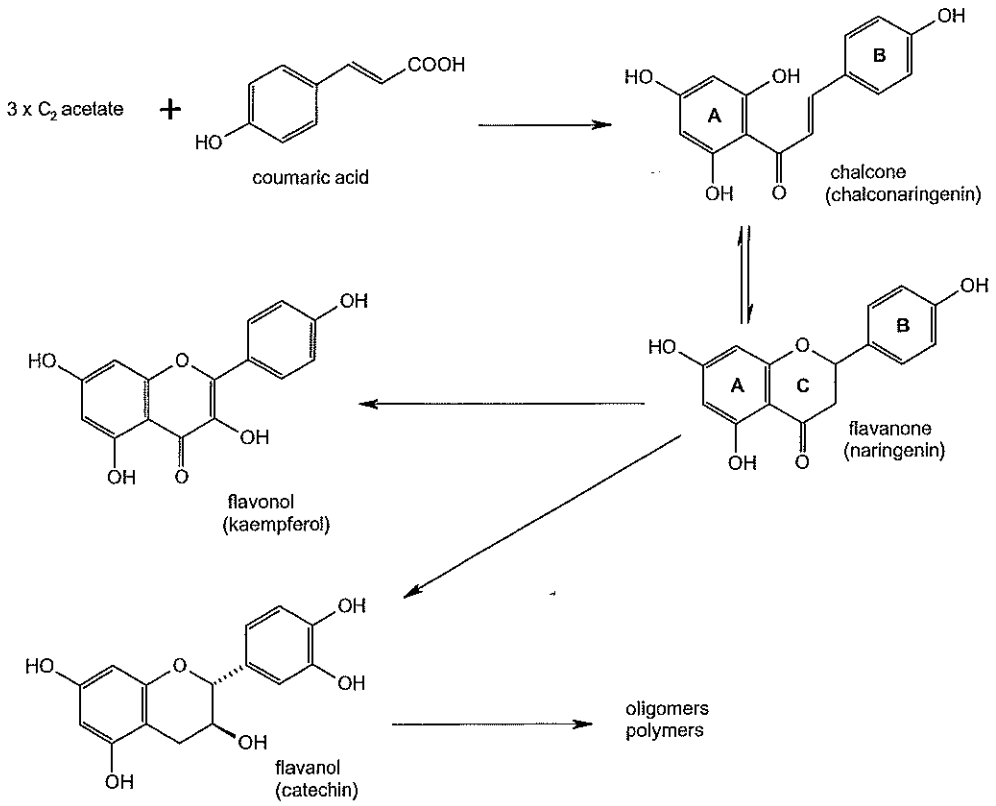


Figure 5: Polyphenol biosynthetic pathway.

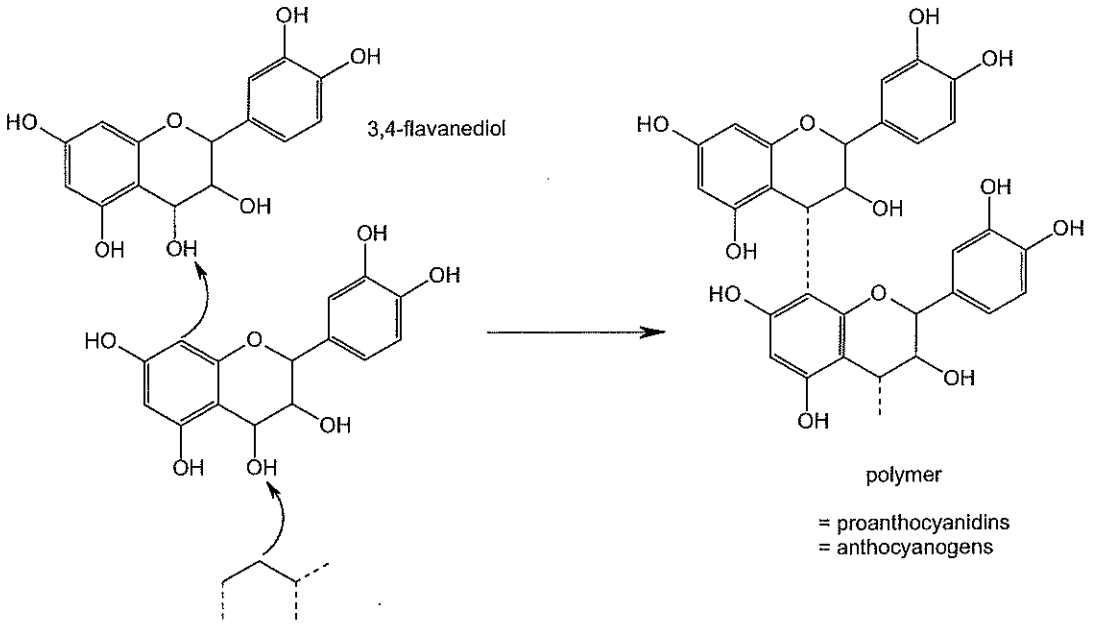


Figure 6: Flavanol polymerisation.

Chalcones are the first intermediates in the flavonoid biosynthetic pathway (Figure 5), after the reaction between coumaric acid and three acetate units catalysed by the enzyme *chalcone synthase*. Further addition of prenyl or geranyl can lead to prenylated chalcones. The flavanone structure is obtained through chalcone isomerisation by the enzyme *chalcone isomerase*. Flavanone oxidation leads to flavonols while reduction leads to flavanols (Stevens *et al.*, 1998).

Flavanol polymerisation can also occur leading to the well-known proanthocyanidins or anthocyanogens. In this

case, a bond is formed between the C_8 of the A ring and the C_4. Small polymers with less than 10 units are usually called oligomers and long polymers are known as tannins (Figure 6).

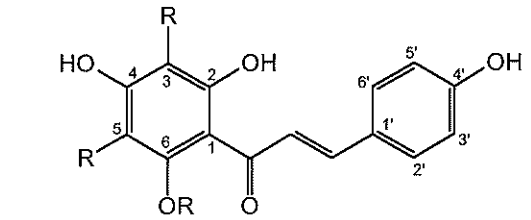
Prenylchalcones

As shown above, chalcones are open flavonoid structures. In hops, the chalcone A ring often bears a prenyl-group substituent (De Keukeleire *et al.*, 1999; Stevens *et al.*, 1999). Stevens *et al.* (1997) have identified 9 prenylated flavonoids in hop, six of which are chalcones. Xanthohumol alone represents more than 80% of the hop prenylflavonoids (Figure 7). In some hop samples, over 6000 ppm (0.6%) has been found (Stevens *et al.*, 1999). Moreover, xanthohumol is believed to give rise to oestrogenic activity (De Keukeleire *et al.*, 1999).

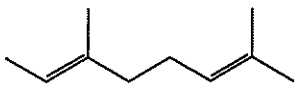
Analysis of several beers showed concentrations ranging from 0 to 4 ppm (the highest value was observed for a Stout beer). Since hop is the sole source of prenylated flavonoids in beer, a clear relationship can be established between their concentration and the hopping rate. When the xanthohumol-to-isoxanthohumol ratio is high in a beer, this indicates a short period of contact between the hop and the boiling wort, as isomerisation occurs during this period. The use of CO_2 hop extracts leads to xanthohumol concentrations close to 0 (Stevens *et al.*, 1999).

Flavanoids

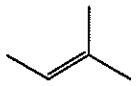
The flavanoid family is extremely diversified (Figure 8). There is a 2,3 double bond conjugated with a 4-oxo function in flavones and isoflavones, a dihydroxyl structure on carbons 3 and 4 in 3,4 flavanediols, only one substituent at position 3 in flavan-3-ols, and a carbonyl at position 4 in flavanones. The B ring can also be moved to position 3 or 4 as in the case of isoflavones and neoflavanes.



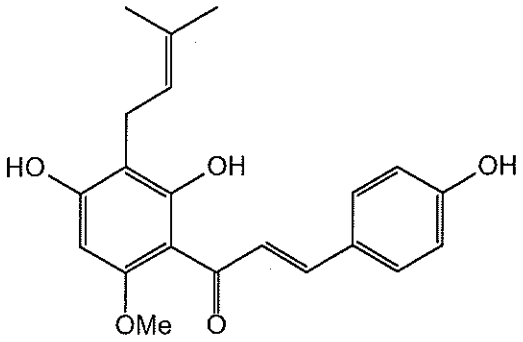
R = H, methyl, isoprenyl, geranyl, ...



geranyl group



isoprenyl group



Xanthohumol = 85 – 90% of hop prenylchalcones

Figure 7: Structures of hop prenylchalcones.

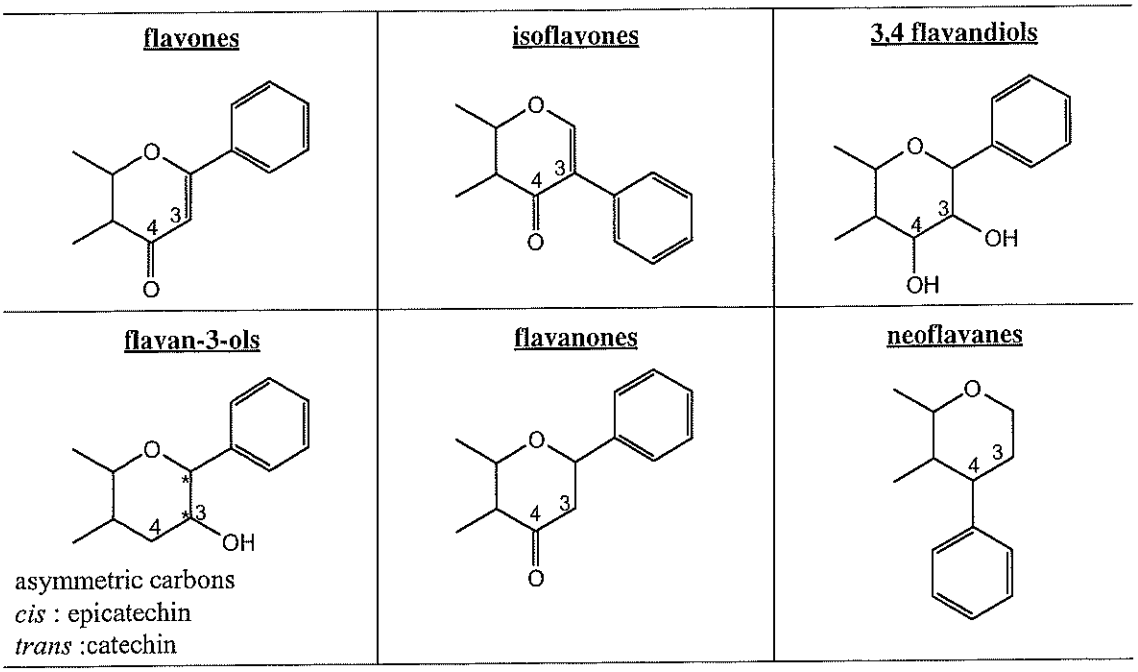


Figure 8: Structures of flavanoids.

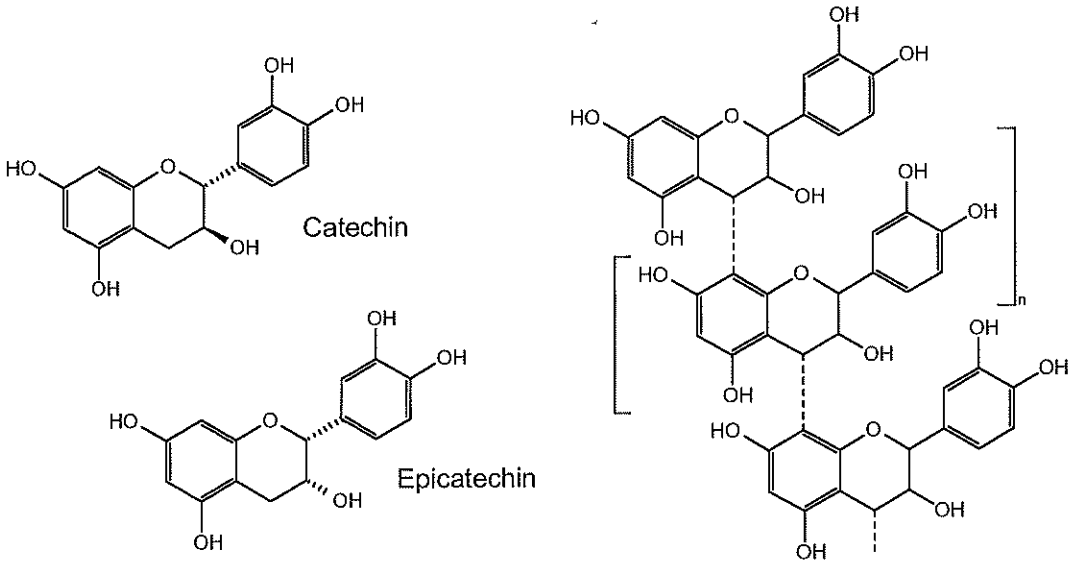


Figure 9: Structure of oligomeric flavanoids and of monomers (+)-catechin or (-)-epicatechin.

In hop, flavanoids are mainly represented by flavan-3-ols: catechin, epicatechin and anthocyanogens (McMurrough & Hennigan, 1984; De Keukeleire *et al.*, 1999). They represent 1 to 5% of the dry weight and they have been studied in depth by McMurrough and Hennigan in 1984 (Figure 9). These authors isolated from hop (*Bullion* variety) six flavanoid subfractions: monomers, dimers, trimers, and three polymerised fractions with respective Apparent Polymerisation Indices of 3.76 - 4.71 and 11.7.

Table 7: Hop flavanoid content and apparent polymerisation indices of subfractions.

Fraction	Abundance *(% total polyphenols)*	Apparent Polymerisation Indice
Monomers	1.4	1.00
Dimers	1.5	1.94
Trimers	0.4	3.13
Polymers I	4.0	3.76
Polymers II	83.4	4.71
Polymers III	8.3	11.7

As shown in Table 7, simple flavanols (monomers, dimers, and trimers) represent only 3% of the hop

flavanoids. Two monomers can be found: catechin and epicatechin. Proanthocyanidin B_1 (catechin + epicatechin) and B_3 (2 catechins) constitute the majority of the dimer fraction while the most abundant trimer is proanthocyanidin C_2 (3 catechins). The oligomer fraction with an API of 4.7 is by far the most abundant representing up to 80% of the flavanoid extract (McMurrough & Hennigan, 1984).

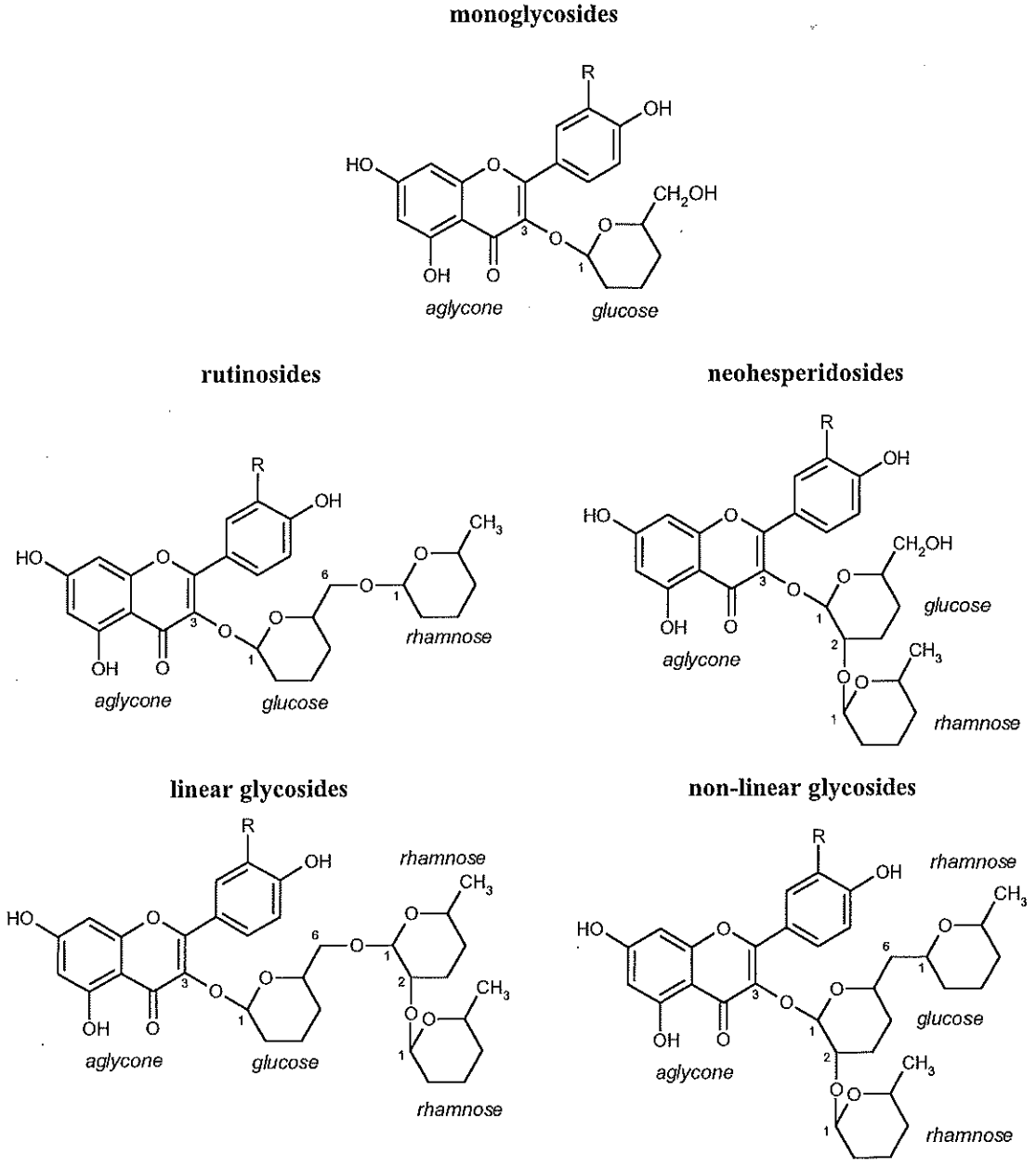


Figure 10: Structure of monoglycosides, diglycosides and triglycosides found in hop.

Flavonols

Flavonols constitute only 0.1 to 0.4% of the hop weight. In flavonols, the additional hydroxyl function on carbon 3 allows binding of different carbohydrate residues (Figure 11) (McMurrough *et al.*, 1982; Sägesser & Deinzer, 1996). Sixteen flavonol glycosides have been detected in hops (Vancraenenbroek *et al.*, 1969; McMurrough *et al.*, 1982; Sägesser & Deinzer, 1996). Most of them were characterised as quercetin and kaempferol mono, di and triglycosides (Figure 10). The aglycones represent respectively 0.08 - 0.16% and 0.03 - 0.1% of hop weight. Only small amounts of myricetin have been found in hops (McMurrough, 1981).

The four main glycosides are the quercetin and kaempferol β-3-glucosides and β-3-rutinosides (1 glucose+1 rhamnose) (McMurrough, 1981). Although boiling allows extraction of 90% of the flavonol glycosides, they are found in very low amounts in wort (1.2 ppm quercetin and 2 ppm kaempferol) and beer (0.5 ppm quercetin and 0.95 ppm kaempferol). Despite their very high antioxidant activity, flavonols are thus expected to have a low impact on hop and wort reducing power (McMurrough *et al.*, 1982; Sägesser & Deinzer, 1996). According to vanSumere *et al.* (1987), great variations in glycoside ratios exist between varieties, making it possible to identify cultivars on this basis.

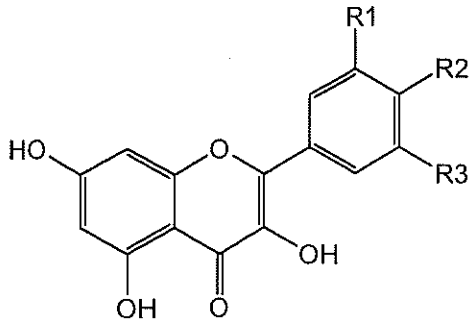


Figure 11: Flavonol structure; R2 = OH : kaempferol, R1 = R2 = OH : quercetin, R1 = R2 = R3 = OH : myricetin.

CONCLUSION

We have shown the major contribution of polyphenols to hop reducing power. Being the most abundant polyphenols in hops, flavanoid concentrations explain the differences observed between hop cultivars. Further work should make it possible to identify the flavanol subfractions, their concentrations in hop and their influence on hop reducing power. On the other hand, hop prooxidants should also be studied to determine their negative effects on wort an beer oxidation.

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