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Release of hydroxycinnamic acids and formation of flavour-active volatile phenols during the beer production process

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door

Nele VANBENEDEN

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LIST OF ABBREVIATIONS

°P	degrees Plato; 1 °P = 1 %(w/w) soluble extract
4EG	4-ethylguaiacol
4EP	4-ethylphenol
4NPF	4-nitrophenyl ferulate
4VG	4-vinylguaiacol
4VP	4-vinylphenol
alc.	alcohol content
AO	antioxidant activity
ASTM	American Society for Testing and Materials
AX	arabinoxylans
AZCL	azurine cross-linked
BET	best estimate value threshold
Da	Dalton (1/12 of the mass of carbon 12)
dm	dry matter
DMSO	dimethylsulfoxide
EBC	European Brewery Convention
EBU	European Bitterness Units
EC	Enzyme Commission
EEC	European Economic Community
Eorig	original extract
EU	enzyme unit
Ea	apparent extract
FA	ferulic acid
FU	flavour units
GC-MS	gas-chromatography with mass spectrometry
GH	glycoside hydrolase
GRAS	generally regarded as safe
HCA’s	hydroxycinnamic acid and its methoxylated derivatives

HPLC	high performance liquid chromatography
HPAEC-PAD	high performance anion exchange chromatography with pulsed amperometric detection
HS-GC-FID	headspace gas-chromatography with flame ionisation detection
LLOQ	lowest limit of quantification
MES	2-(N-morpholino)ethanesulfonic acid
MFA	methyl ferulate
MLR	multiple linear regression
MpCA	methyl *p*-coumarate
MSA	methyl sinapate
m/z	mass to charge ratio
ND	not detectable
NIST	National Institute of Standards and Technology
OD	optical density
PAD1/Pad1	phenylacrylic acid decarboxylase (gene and enzyme)
pCA	*p*-coumaric acid
POF	phenolic off-flavour
ppb	parts per billion (corresponding to µg/L)
ppm	parts per million (corresponding to mg/L)
PVPP	polyvinylpyrrolidone
RP-HPLC-ECD	reversed phase HPLC with electrochemical detection
rpm	rotations per minute
RT	retention time *or* reverse transcriptase
SA	sinapic acid
SGD	*Saccharomyces* Genome Database (www.yeastgenome.org)
TFA	trifluoroacetic acid
Trizma	tris(hydroxymethyl)-amino-methane
qPCR	quantitative polymerase chain reaction
WEAX	water-extractable AX
WUAX	water-unextractable AX

SAMENVATTING

De meeste vluchtige fenolen in bier zijn afkomstig van de grondstoffen die gebruikt worden tijdens het brouwproces. De aroma-actieve fenolen 4-vinylfenol (4VP) en 4-vinylguaiacol (4VG) worden echter hoofdzakelijk gevormd tijdens de fermentatie door brouwersgist. De aanwezigheid van deze componenten is ongewenst in pilsbier. Hierdoor werden ze historisch beschouwd als *off-flavours*. Ze maken echter essentieel deel uit van het karakteristieke aroma van vele blonde speciaalbieren, witbieren en andere tarwebieren waar ze een kruidig of gerookt aroma aan de betreffende bieren verlenen. De laatste jaren worden deze smaakcomponenten in toenemende mate prominent aangetroffen in het aroma van Belgische speciaalbieren. Hoewel sommige brouwers de aanwezigheid van deze componenten duidelijk wensen in (sommige van) hun bieren, brengen ze het aromaprofiel van vele andere bieren, door hun soms overheersende aroma, uit balans. Er is weinig geweten over de dynamiek van de vrijzetting van hun precursoren (de hydroxykaneelzuren *p*-coumarinezuur en ferulinezuur) tijdens het brouwproces en de interactieve rol die de mouteigen enzymen hierbij kunnen spelen. Karakterisering van de betrokken enzymen en onderzoek naar de invloed van de procesparameters tijdens de bierproductie op de vrijstelling van de precursoren en de daaropvolgende omzetting tot vluchtige fenolen is nodig om de brouwers toe te laten de gehaltes aan hydroxykaneelzuren en vluchtige fenolen te beheersen afhankelijk van de beoogde bierstijl en om een constante batch-to-batch kwaliteit te kunnen afleveren. Vooreerst werd een analytische methode (RP-HPLC-ECD) op punt gesteld en gevalideerd die gebruikt kan worden voor de bepaling van deze componenten en hun precursoren in wort en bier. Deze methode werd gebruikt voor een grootschalige analyse van Belgische speciaalbieren om het voorkomen van vluchtige fenolen en hun precursoren in verschillende bierstijlen in kaart te brengen. Geur- en smaakdrempels werden bepaald in water en in verschillende biermedia om de impact van deze aromacomponenten op het bieraroma te bepalen. Vervolgens werd de evolutie van hydroxykaneelzuren en vluchtige fenolen opgevolgd tijdens het volledige bierproductieproces om realistische controlepunten te identificeren. Er werd een grote variabiliteit in het fenolzuurgehalte tussen verschillende moutvariëteiten gevonden. Ook werden verschillen aangetroffen tussen gelijke moutvariëteiten afkomstig van verschillende mouterijen. Dit wijst op het belang van de keuze van een gepaste moutvariëteit wanneer men het uiteindelijke gehalte aan vluchtige fenolen in bier wenst te beheersen. De esterase- en endoxylanase-activiteit van de mout alsmede het gehalte aan estergebonden

hydroxykaneelzuren in wort werden geïdentificeerd als verklarende factoren voor het finale gehalte aan vrije hydroxykaneelzuren in wort. Eveneens na de keuze van de gepaste moutvariëteit is er nog ruimte voor de beheersing van de uiteindelijke fenolzuurconcentratie in wort. Doordat hydroxykaneelzuren deels worden vrijgezet door een enzymatische reactie tijdens het brouwen, kan men door variatie van allerhande procesparameters in de brouwzaal, het gehalte aan hydroxykaneelzuren in bier beïnvloeden. Vrije hydroxykaneelzuren in wort zijn beschikbaar voor decarboxylatie tot aroma-actieve fenolen. Deze decarboxylatie kan zowel thermisch als enzymatisch geïnitieerd worden. Voor de soms hoge gehaltes aan vluchtige fenolen die vaak aangetroffen worden in blonde speciaalbieren, volstaat thermische decarboxylatie tijdens bvb. het wortkoken, niet als verklarende factor. Hiervoor is een fermentatie met een Pad1-actieve giststam noodzakelijk. Deze enzymactiviteit (*phenylacrylic acid* decarboxylase) werd met een grote frequentie aangetroffen in hoge gisten. De mate waarin verschillende giststammen in staat zijn om hydroxykaneelzuren te decarboxyleren verschilt echter sterk. Dit maakt dat de keuze van een geschikte giststam zeer belangrijk bij de optimalisatie van vluchtige fenolen in bier. De activiteit van het Pad1-enzym is maximaal naar het einde van de gisting toe, wanneer alle vergistbare suikers bijna verbruikt zijn. Dit suggereert dat *PAD1* betrokken is in het stressmetabolisme van gist dat geactiveerd wordt bij substraatuitputting. Er was een duidelijk verband tussen de fenolzuurconcentratie van wort en de uiteindelijke concentratie aan vluchtige fenolen in bier, wat er op duidt dat de optimalisatie van het fenolzuurgehalte in wort een realistische optie is wanneer men het gehalte aan vluchtige fenolen wenst te beheersen. Fermentaties op pilootschaal toonden aan dat technieken geassocieerd met modern gistmanagement in cilindroconische fermentatietanken, een grote invloed hebben op het gehalte aan 4VG in bier. Finaal werden de oorzaken van de daling van de 4VG concentratie tijdens de bierbewaring achterhaald. Een nieuwe vanille-achtige component in bier, apocynol, werd geïdentificeerd als het belangrijkste degradatieproduct van 4VG tijdens de bierveroudering. Deze component wordt gevormd door de zuur-gekatalyseerde hydratatie van de dubbele binding in de zijketen op de aromatische ring van 4VG. Wanneer er zuurstof aanwezig is in de flessenhals tijdens de bierbewaring kan 4VG ook deels geoxideerd worden tot vanilline. Beide reactiemechanismen kunnen aanleiding geven tot het vanille-achtig aroma dat ontstaat in sommige bieren tijdens de bewaring.

ABSTRACT

Among the flavour-active volatile phenols in beer, most of them originate from the raw materials used in the brewing process. Only some of them can be formed by yeast activity, namely 4-vinylguaiacol (4VG) and 4-vinylphenol. The presence of these volatile phenolic compounds is considered undesirable when present in excessive concentration in bottom-fermented pilsner beers, hence the term "phenolic off-flavour". It is attributed to beers with a strong medicinal, clove-like aroma. Despite being historically catalogued as an off-flavour, these compounds are known to be essential flavour contributors to the characteristic aroma of Belgian white beers, German Weizen beers and Rauch beers. In recent years, volatile phenolic flavour compounds have been increasingly encountered in Belgian specialty beers. While some brewers wish for a clear phenolic note in (some of) their beers, others do not aim for their, sometimes overwhelming, presence in the taste pallet of beer. Little is known of the dynamics behind the release of their precursors (the hydroxycinnamic acids (HCA's) ferulic acid and *p*-coumaric acid) during brewing, the interactive role of native barley enzymes underlying this release and the subsequent decarboxylation to the flavour-active compounds during wort fermentation. Also the causes of the temperature dependent decrease of 4VG during beer ageing need to be elucidated. The suitability of a simple and rapid isocratic RP-HPLC method with amperometric detection for the simultaneous detection and quantification of HCA's and their corresponding aroma-active volatile phenols in wort and beer is developed and validated. The method was used to perform an extensive survey on the occurrence of HCA's and volatile phenols in a range of beer styles. Odour and flavour thresholds of 4VG determined in a diverse range of beer styles confirmed the contribution of 4VG to the overall flavour perception of many top-fermented specialty beers. HCA's and volatile phenols are monitored throughout the beer production process to identify realistic control points for the final volatile phenol level in beer. A large variability in HCA's content between different barley malt varieties and their corresponding worts was observed. Differences were also found between free HCA's levels from identical malt varieties originating from different malthouses. This demonstrates the importance of selecting a suitable malt variety. It was shown that only a small part of the HCA's in malt is transferred to wort during mashing, while the lion's share remains in the spent grains. Free HCA's in wort are both water-extracted and enzymatically released by cinnamoyl esterase activity. Esterase activities clearly differ between different barley malt varieties, as do other

arabinoxylan-degrading enzyme activities. The release of ferulic acid during mashing did not only depend on the esterase activity, but also on the amount of esterbound ferulic acid initially present in the wort and on the endoxylanase activity. Apart from the choice of a suitable barley malt variety, final HCA's concentrations in wort are also seriously affected by brewhouse operations. A clear difference in temperature and pH dependence between the release of the water-extracted and enzymatically hydrolysed fraction was found. In contrast to the water-extracted fraction, the hydrolysis of esterbound ferulic acid is subject to close technological control. To contribute to the odour and taste pallet of specialty beers, HCA's have to be decarboxylated to the corresponding volatile phenols. Concerning thermal decarboxylation in pilsner beer, the combined time of wort boiling, transfer, whirlpool and pasteurisation times can give rise to the 4VG concentrations observed in the survey. However, the high concentrations often encountered in blond and dark specialty beers must originate from the enzymatic decarboxylation of HCA's by *Saccharomyces cerevisiae* yeast strains during wort fermentation. The differences in volatile phenol content in top-fermented specialty beers are reflected in the high incidence of Pad1 phenotype among top-fermenting brewing yeasts strains and the observed differences in Pad1 activity between different brewing strains. Clearly, the first means for optimising the volatile phenol content in beer is the choice of a suitable yeast strains. During alcohol fermentation, it was clearly shown that the majority of 4VG is formed during the second half of the fermentation process when nearly all fermentable sugars were consumed. This suggests that the Pad1 enzyme might be involved in the course of events triggered upon the stress presented by nutrient depletion. Clearly, elevated initial FA concentration in wort led to an increase in the 4VG content of beer. This validates the possibility of optimising the final volatile phenol content in beer by controlling the release of HCA's in the brewhouse. Pilot-scale fermentation experiments showed that yeast management systems frequently encountered with cilindroconical tank fermentations significantly affect 4VG formation during wort fermentation. Finally, the decrease in 4VG during beer ageing was examined. Two reaction mechanisms explaining the decrease of 4VG during beer ageing were identified. A new vanilla-like compound in beer, apocynol, was identified as the main degradation product. Apocynol is formed by an acid-catalysed hydration of the double bond of the vinyl side chain of 4VG. In the presence of oxygen, substantial amounts of vanillin were also detected. Since both apocynol and vanillin have a clear vanilla-like aroma, the decrease of 4VG during beer ageing may impart a shift from a clove-like aroma in a fresh specialty beer to a more sweet, vanilla-like flavour impression of aged specialty beer.

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CHAPTER I

FLAVOUR-ACTIVE VOLATILE PHENOLS IN BEER: A LITERATURE REVIEW

This chapter summarises the current knowledge about volatile phenols and hydroxycinnamic acids in fermented beverages and, more specifically, in beer. In the first part, possible routes for formation of volatile phenols are highlighted. The next part discusses arabinoxylans and arabinoxylan degrading enzymes, while the last paragraph gives a more detailed literature review about the occurrence of hydroxycinnamic acids in cereal grains.

I.1. VOLATILE PHENOLS IN FERMENTED BEVERAGES

I.1.1. Introduction

Phenols in beer are present either in monomeric or in polymeric form. Phenolic monomers in beer include flavonoids such as flavanols (e.g. catechin) and flavonols (e.g. quercetin, myricetin) (Jandera *et al.*, 2005; Rehova *et al.*, 2004), phenolic acids (McMurrough *et al.*, 1984; Montanari *et al.*, 1999; Nardini *et al.*, 2004) and volatile phenols (McMurrough *et al.*, 1996; Tressl *et al.*, 1976; Wackerbauer *et al.*, 1982a). Phenolic acids are simple monocyclic acids and comprise the hydroxy derivatives of benzoic and cinnamic acid. Almost 20 different derivatives of benzoic (e.g. vanillic acid, gallic acid, syringic acid) and cinnamic acid (e.g. *p*-coumaric acid, ferulic acid, sinapic acid) (figure I.1) can be detected in beer (Floridi *et al.*, 2003). Most of them have high threshold values and do not affect the aroma of beer. However, they are appreciated for their antioxidant (AO) activity. Recently, phenolic acids present in beer in esterbound form to arabinoxylans (paragraph I.2.) have also become a topic of interest because of their potential AO capacity (Szwajgier *et al.*, 2005b). Beer may be an important source of daily bound phenolic acid intake in western diets. Information about the content of free *versus* bound phenolic acids in beer is scarce (Nardini *et al.*, 2004).

Among the flavour-active volatile phenols guaiacol, phenol, vanillin, acetovanillon, eugenol, 4-vinylsyringol, 4-vinylguaiacol and 4-vinylphenol, have been detected in beer (Tressl *et al.*, 1976). Most of these simple phenolic compounds originate from the raw materials used in the brewing process or from brewing water contaminated with, for example, chlorophenols. Only some of them can be formed by yeast activity, namely 4-vinylguaiacol (4VG) and 4-vinylphenol (4VP) (figure I.1). The presence of these volatile phenolic compounds is considered undesirable when present in excessive concentration in bottom-fermented pilsner beers, hence the term “phenolic off-flavour” (POF) (Thurston and Tubb, 1981). It is attributed to beers with a strong medicinal or clove-like aroma. Despite being historically catalogued as an off-flavour, these compounds are known to be essential flavour contributors to the characteristic aroma of Belgian white beers (made with unmalted wheat), German Weizen beers (made with malted wheat) and Rauch beers (Back *et al.*, 2000; Kieniger *et al.*, 1984; NarziB *et al.*, 1990; Tressl *et al.*, 1976; Wackerbauer *et al.*, 1982b).

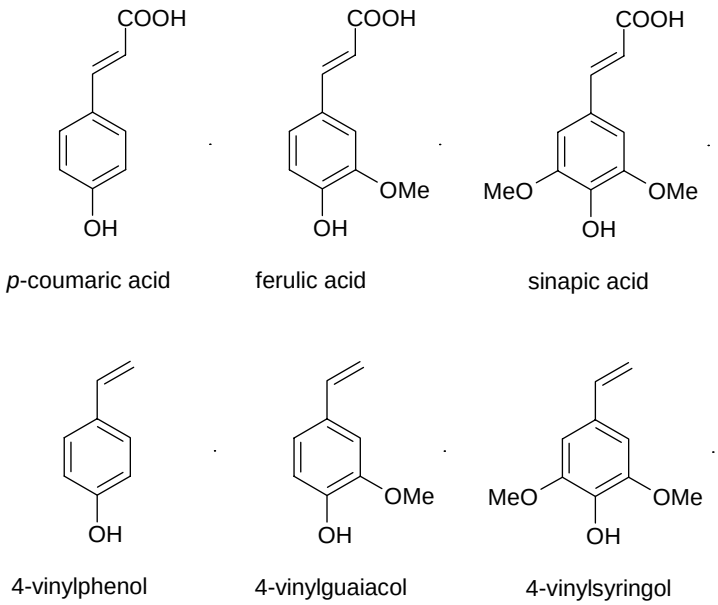


Figure I.1. *Structure of the hydroxycinnamic acids* p-*coumaric acid, ferulic acid, sinapic acid and their corresponding decarboxylated volatile phenols 4-vinylphenol, 4-vinylguaiacol and 4-vinylsyringol*

4VG is also a key flavour compound in coffee (Dorfner *et al.*, 2003). The 4VG content in Robusta coffee is reported to be much higher than in Arabica coffee. It might be responsible for the smoky phenolic odour note, which is more intense in the Robusta coffee brew (Semmelroch *et al.*, 1996). Vinylphenols have been reported as potential off-flavour compounds in other non-alcoholic drinks like apple juice (Donaghy *et al.*, 1999) and orange juice (Fallico *et al.*, 1996; Lee *et al.*, 1990; Marcotte *et al.*, 1998; Naim *et al.*, 1993; Naim *et al.*, 1997; Peleg *et al.*, 1992; Rouseff *et al.*, 1992; Walsh *et al.*, 1997) where they impart an old or rotten fruit aroma. They also contribute to the aroma of various other alcoholic drinks like whisky (Lee *et al.*, 2000; van Beek *et al.*, 2000) and sherry (Dominguez *et al.*, 2002; Tressl *et al.*, 1976). In wine, both vinylphenols (4VP and 4VG) and their ethyl analogues (4-ethylphenol and 4-ethylguaiacol) have been detected and are important contributors to wine aroma (Carrillo *et al.*, 2006; Chatonnet *et al.*, 1993; Diez *et al.*, 2004; Ferreira *et al.*, 1997; Lopez *et al.*, 2002; Mejias *et al.*, 2003; Pollnitz *et al.*, 2000; Shinohara *et al.*, 2000). Red wines are generally characterised by higher concentrations of ethylphenols than vinylphenols while white wines are characterised by low levels in ethylphenols and high levels of vinylphenols. Volatile phenols in wine are usually present in concentrations varying from a few dozen to several hundreds ppb. Amounts of a few ppm can be observed in irremediably defective wines. 4VP, even if under the sensory threshold, is deemed to negatively affect and mask the fruity scent of white wines conferring odours resembling “band aid”. However, it is considered less negative when combined with 4VG. 4VG is known to contribute to the

typical spicy note of Gewuertztraminer wines. 4-Ethylphenol (4EP) has a stable, horse sweat, leather-like odour, while 4-ethylguaiacol (4EG) is also described as sweet. Blends of ethylphenols in red wine give stable and animal-like odours (Larcher *et al.*, 2007).

Steinke and Paulson (1964) first identified the phenolic acids *p*-coumaric acid (4-hydroxycinnamic acid) (pCA) and ferulic acid (4-hydroxy-3-methoxycinnamic acid) (FA) as the precursors for 4VP and 4VG, respectively. Phenolic acids (i.e. hydroxycarboxylic acids with phenolic hydroxyl groups), more specifically hydroxycinnamic acids (HCA's) (figure I.1) like pCA, FA and sinapic acid (4-hydroxy-3,5-dimethoxycinnamic acid) (SA), are mainly associated with polysaccharides in the plant cell wall. In cereal grain, they are mainly esterified with arabinoxylans (AX). AX are important structural carbohydrates in the husk, pericarp, aleurone and endosperm of cereal grains. They consist of β-(1-4)-xylans in which xylose residues are substituted with arabinose at C2 and/or C3. Cinnamoyl groups can be attached to the arabinofuranosyl residues at the O5. AX are high molecular weight, partly water-soluble polymers. During the brewing process, they are extracted and solubilised by AX-hydrolases from the malt into the wort (Debyser *et al.*, 1997a). FA, pCA and SA can be released as free acids by cinnamoyl esterase activity during mashing. Cinnamoyl esterases have been reported natively in barley (Humberstone and Briggs, 2002) and barley malt (Sancho *et al.*, 2001; Sun *et al.*, 2005). The flavour-inactive phenolic acids, having a flavour threshold as high as 600 ppm (Meilgaard, 1975), can be decarboxylated to the highly flavour-active volatile phenols 4VP and 4VG in two ways (Wackerbauer *et al.*, 1977; Wackerbauer *et al.*, 1982a): (1) by thermal impact during high temperature treatments in the beer production process, or (2) by enzymatic decarboxylation during fermentation by top-fermenting yeasts strains or contaminating micro-organisms.

I.1.2. Thermal decarboxylation

4VG in beer can arise from the thermal decarboxylation of FA during high temperature treatments during the malt and beer production process like kilning, wort boiling, whirlpool holding, wort transfer times and beer pasteurisation. Since mashing temperatures are inadequate for the thermal decarboxylation of FA, no 4VG can be detected in unboiled wort. During wort boiling, the 4VG concentration will increase with increasing boiling times. Higher levels of precursor molecules will lead to higher 4VG levels after boiling, but only intensive and long boiling regimes will give rise to phenolic flavour compounds exceeding the flavour threshold (Coghe *et al.*, 2004a; McMurrough *et al.*, 1996; Ogane *et al.*, 2006).

Thermal fragmentation of FA can also occur during the production of highly coloured specialty malts. 4VG was detected in extracts from roasted malt and roasted barley. However, since roasted barley or roasted malt make up only a small proportion of the grist, this will only have a minor effect on the final content of 4VG in beer (Coghe *et al.*, 2004b; Kieniger *et al.*, 1977; Samaras *et al.*, 2005).

Fiddler *et al.* (1967) studied the thermal decomposition of FA in solid state by thermal gravimetric analysis. Decomposition started at 200 °C. In the first stage of the decomposition, only 4VG was found. At temperatures exceeding 340 °C, unsubstituted, 4-methyl and 4-ethylguaiacol were formed. In air atmosphere, also vanillin, acetovanillon and vanillic acid were formed. Since the labile double bond of 4VG is readily susceptible to air oxidation, 4VG is considered to be the precursor of these oxygenated products. The rate of the reaction during the first phase is accelerated by oxygen indicating that the decarboxylation is probably a radical reaction. Hashidoko and Tahara (1998) investigated the stereochemistry of the pyrolysis products of pCA. On thermal decarboxylation, a geometric scramble on the vinyl group occurred and no selective hydrogen incorporation was observed.

I.1.3. Enzymatic decarboxylation

I.1.3.1. Enzymatic decarboxylation by Saccharomyces *yeasts*

Phenolic acids can be enzymatically decarboxylated during fermentation by phenylacrylic acid decarboxylase activity of top-fermenting yeasts strains (Pad1-enzyme). Bottom-fermenting yeasts strains cannot form 4VG (McMurrough *et al.*, 1996; Narziβ *et al.*, 1990; Perpete *et al.*, 2001). On the contrary, all *Saccharomyces diastaticus* yeast strains have been invariably associated with POF production (Russell *et al.*, 1983; Stewart *et al.*, 1983; Villareal *et al.*, 1986). Among *Saccharomyces cerevisiae* wine yeasts, a high frequency of strains having the ability to decarboxylate pCA and FA was found. Both Grando *et al.* (1993) and Shinohara *et al.* (2000) found that the majority of yeasts isolated from grape musts and winemaking environments were POF-producing strains. Pad1 confers resistance to HCA's. The activity might confer upon the saprophyte *Saccharomyces cerevisiae* a selective advantage when growing on decaying fruit, a rich source of HCA's. Also, their decarboxylation to styrenes perturbs isoflavonoid and lignin biosynthesis thereby facilitating pathogen ingress (Clausen *et al.*, 1994).

Cinnamic acid and cinnamic acid derivatives occur in plants and fruits providing a natural protection against infections of pathogenic microorganisms. The non-ionised form of the HCA's can enter the cell. Since their pKa's are significantly below the pH of the cytosol, they dissociate in the cytoplasma. The released protons are pumped out at the expense of ATP. Accumulation of the resulting anions is an additional mechanism of toxicity (Narendranath *et al*., 2001). Both pCA and FA have been found to increase the lag phase of *Saccharomyces cerevisiae*. The degree of inhibition is inversely related to the degree of the polarity of the compound. FA, the most non-polar, was found to be the most inhibitory (Baranowski *et al.*, 1980). Cells grown in the presence of cinnamic acid have been found to exhibit a more active plasma membrane H^+-ATPase (Chambel *et al.*, 1999).

The $Pad1^+$ phenotype is controlled by a single dominant nuclear gene (Goodey *et al.*, 1982). *PAD1* is located at the end of chromosome IV and is not essential for viability (Clausen *et al.*, 1994). The transformation of POF(-) strains with the cloned *PAD1* gene led to the production of an aroma characteristic of a phenolic off-flavour. This observation suggests that the POF(-) phenotype of brewer's yeast is specifically due the absence of a functional *PAD1* gene (Meaden and Taylor, 1991). Although sequence differences have been found, the DNA sequence of the *PAD1* gene is highly conserved among POF(-) strains (Hwang *et al.*, 1992). The *PAD1* gene is steadily transcribed, but the encoded product shows only low activity. *PAD1* mRNA levels during growth are constant and the overexpression of the *PAD1* gene did not show an elevated enzyme activity. This might indicate that post-transcriptional regulation might be critical for the functioning of Pad1 (Clausen *et al.*, 1994; Smit *et al.*, 2003). Pad1-activity is expressed with a variable intensity depending on the yeast strain. The Pad1-enzyme is cytoplasmatic. The activity is inhibited by polyphenolic compounds like catechic tannins (Chatonnet *et al.*, 1993). It shows greater activity under oxygen limitation. Unlike other decarboxylases, the non-oxidative decarboxylase operates in the absence of enzyme-bound cofactors (Larsson *et al.*, 2001).

Yeasts strains possessing Pad1 activity are capable of transforming FA and pCA by non-oxidative decarboxylation. Benzoic acids and caffeic acid cannot be converted. Also certain substitution patterns, e.g. 2.5- and 3.5-, are resistant to decarboxylation by yeast cells (Chatonnet *et al.*, 1993; Goodey *et al.*, 1982; Gramatica *et al.*, 1981; McMurrough *et al.*, 1996). There is some controversy whether or not *Saccharomyces cerevisiae* is able to decarboxylate unsubstituted cinnamic acid to styrene. According to some, substitution of the

aromatic ring with an oxygen-containing group in *para* is a pre-requisite for the decarboxylation reaction (Arfmann *et al.*, 1989; Chatonnet *et al.*, 1993; Gramatica *et al.*, 1981; Huang *et al.*, 1993), but according to others it is not (Clausen *et al.*, 1994; Goodey *et al.*, 1982; Larsson *et al.*, 2001, McMurrough *et al.*, 1996). Depending on whether or not unsubstituted cinnamic acid can be converted, two reaction mechanisms are proposed (figure I.2).

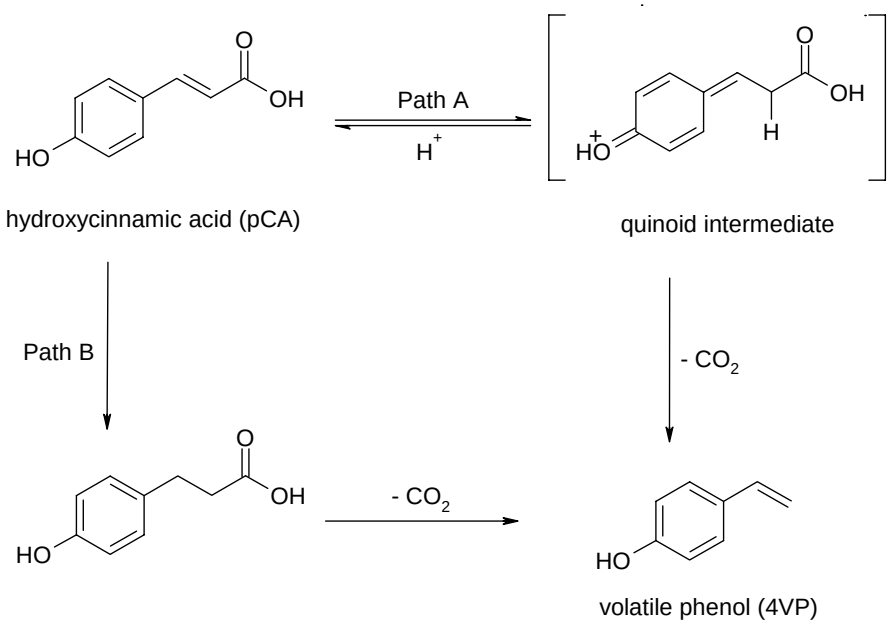


Figure I.2. *Proposed reaction mechanisms for the non-oxidative decarboxylation of the hydroxycinnamic acid pCA by* S. cerevisiae *to the corresponding volatile phenol 4VP through path A (direct decarboxylation through a quinoid intermediate) or through path B (decarboxylation of the reduced form) (Gramatica* et al.*, 1981)*

The first mechanism (path A) proceeds by direct decarboxylation of the hydroxycinnamic acid through a quinoid intermediate. The formation of the quinoid intermediate involves initial enzymatic isomerisation of the HCA's by the addition of a proton from water. The highly labile intermediate is subject to spontaneous decarboxylation. The second mechanism (path B) operates by a preliminary reduction of the side chain double bond. The decarboxylation is stereospecific for the *trans*-configuration and proceeds with the retention of the configuration of the side-chain double bond (Chatonnet *et al.*, 1993; Gramatica *et al.*, 1981; Manitto *et al.*, 1975). Thus, there is a fundamental difference between enzymatic and thermal decarboxylation from a stereochemical specificity perspective.

Hashidoka and Tahara (1998) hypothesised an enzymatic decarboxylation mechanism at the active site of the enzyme for the decarboxylation through the quinoid intermediate pathway (figure I.3). The substrate molecule is initially captured at the carboxylate anion and binds

with a cationic residue (binding site I). The phenolic *para*-OH group of the substrate is captured by another nucleophilic residue of the enzyme (binding site II). The enzyme then abstracts a proton from the bound phenolic OH-group to initiate an electron relay on the conjugated bridge of the cinnamate skeleton. Subsequently, the C8 carbon, appearing as the nucleophilic centre on the quinine form, accepts a proton stereoselectively from a putative acidic proton donor on the enzyme (acidic residue III) to form a *para*-quinone methide intermediate. The second electron relay on the *para*-quinone methide skeleton, starting from the carboxylate anion, takes place immediately, liberating CO_2 and the 4-hydroxystyrene.

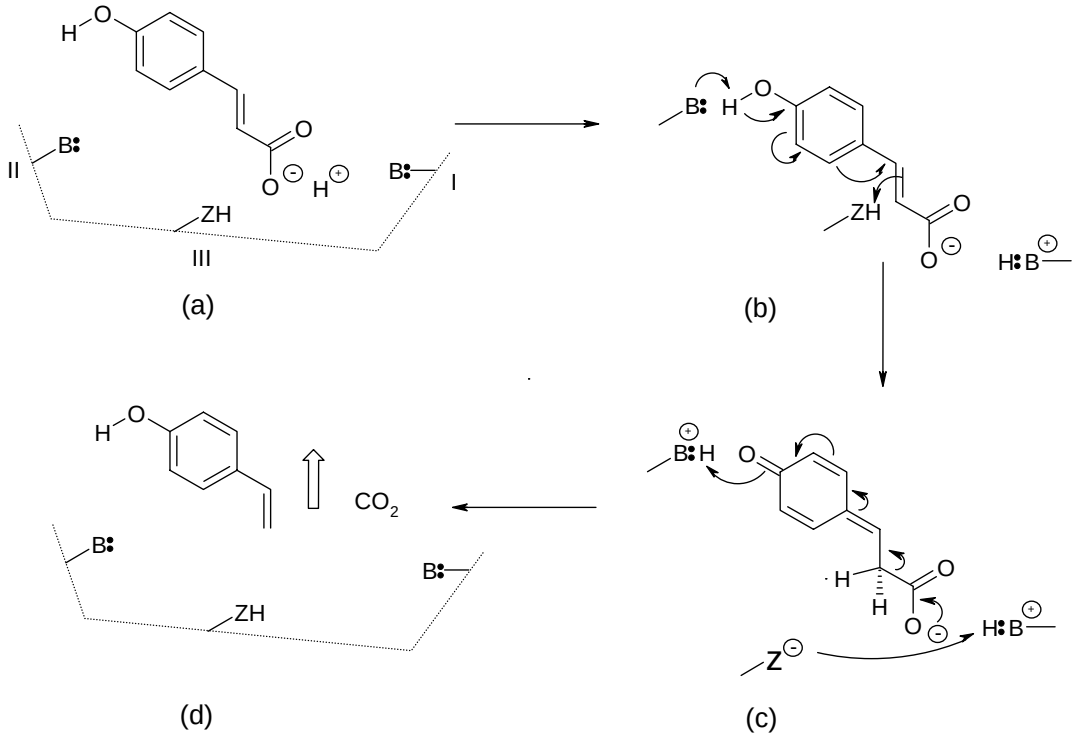


Figure I.3. *Hypothetical process of enzymatic decarboxylation at the active site of a 4-hydroxycinnamate decarboxylase (Hashidoko and Tahara, 1998): (a) the substrate (pCA) captured at the active site (containing two nucleophilic residues (I and II) and one acidic residue (III); (b) an electron relay from the 4-OH group to the C8 nucleophilic center to form a quinoid intermediate; (c) a second electron relay from the carboxylate anion to a electrophilic binding site resulting in cleavage of the C8-C9 bond; (d) liberation of CO_2 and the volatile phenol (4VP)*

*I.1.3.2. Enzymatic decarboxylation by non-*Saccharomyces *yeasts and moulds*

POF has also been associated with a number of wild yeasts, the most important being *Brettanomyces/Dekkera* spp. As already postulated, in wine, both vinylphenols (4VP and 4VG) and their ethyl analogues (4EP and 4EG) have been detected. These ethyl compounds originate from vinylphenol reductase activity, typically associated with *Brettanomyces/Dekkera* spp. (Chatonnet *et al.*, 1992; Edlin *et al.*, 1995). The capacity of

Brettanomyces/Dekkera spp. to form ethylphenols was demonstrated for the first time by Heresztyn (1986) during the fermentation of a grape must. The biosynthesis of ethylphenols by *Brettanomyces/Dekkera* spp. in wine is attributed to the action of two sequential enzymes. First, HCA's are decarboxylated to their vinyl derivatives by an enzyme analogous to the Pad1 enzyme of *Saccharomyces cerevisiae*. Then the vinyl derivative is reduced to the corresponding ethylphenol by vinylphenol reductase activity of *Dekkera/Brettanomyces* spp. *Saccharomyces cerevisiae* yeast strains are not able to execute this reductive step. The ability to form 4EP and 4EG has been shown in *Brettanomyces bruxellensis*, *B. anomalus* and *B. intermedius* isolated during wine fermentations. When wine is stored for long periods in wooden barrels, it becomes nutritionally attractive to slow-growing species. Once the alcoholic and malo-lactic fermentation is completed, *Brettanomyces* spp. grow easily on traces of residual sugars (Suarez *et al.*, 2007). The flavour of the ethyl compounds is identical to that of the corresponding vinyl derivatives but their flavour threshold has been reported to be lower (Chatonnet *et al.*, 1992; Meilgaard *et al.*, 1975). Hence, the reduction may render them more flavour-active and more stable because of the absence of the labile double bond. Edlin *et al.* (1998) have purified a hydroxycinnamate decarboxylase from *B. anomalus*. Enzyme activity was optimal at 40 °C and pH 6. The decarboxylase was able to decarboxylate pCA and FA, but it was inactive towards *ortho*-coumaric acid, *meta*-coumaric acid and cinnamic acid, indicating that the *p*-OH is essential for its activity. Substituting the *p*-OH with a methoxy-group also inhibited the activity. The enzyme activity was shown to be constitutive and substrate inducible (i.e. the enzyme activity increases when cells are grown in the presence of increasing substrate concentrations). Moreover, the decarboxylase of *Brettanomyces/Dekkera* spp. is not inhibited by polyphenolic compounds of red wines (procyanidins and catechins) while these compounds do inhibit the decarboxylase of *S. cerevisiae* (Chatonnet *et al.*, 1993). Dias *et al.* (2003a) examined the conversion of pCA in *Dekkera bruxellensis* during wine fermentation. The production of 4EP occurred roughly between mid-exponential growth phase and the beginning of the stationary phase. The production of 4VP was detected only in the beginning of the growth phase in low amounts because it was rapidly reduced. 4EP production was observed only after complete fermentation of the grape juices by *S. cerevisiae*.

Other wild yeasts that have been associated with POF production, are *Debaryomyces hansenii*, *Candida* spp., *Torulaspora delbreuckii*, *Pichia* spp., *Hanseniaspora uvarum*, *Kluyveromyces*, *Kloeckera apiculata*, *Hansenula anomala*, *Schizosaccharomyces pombe*,

Zygosaccharomyces baillii and *Rhodotorula* spp. (Chatonnet *et al.*, 1992; Donaghy *et al.*, 1999; Mathew *et al.*, 2007; Rosazza *et al.*, 1995; Shinohara *et al.*, 2000; Suezawa *et al.*, 2007). Certain *Candida* spp. and *Pichia guilliermondii* have also been found to be able to produce ethylphenols (Dias *et al.*, 2003b). Several phytopathogenic filamentous moulds, either storage or field fungi of barley (e.g. *Penicillium* spp., *Aspergillus* spp.; *Fusarium* spp.), are also known to be able to decarboxylate HCA's (Rosazza *et al.*, 1995). However, it is unlikely that they affect the phenolic flavour profile of beer since they are eliminated during the malting process.

I.1.3.3. Enzymatic decarboxylation by bacterial wort and beer contaminants

Many bacteria can convert HCA's. Phenylacrylic acid decarboxylase activity is generally higher in bacteria than in *S. cerevisiae*. Bacterial decarboxylase genes (PADC from *Bacillus subtilis* and PDC from *Lactobacillus plantarum*) have been cloned in *S. cerevisiae* in an attempt to develop wine yeast starter strains with an optimised decarboxylation activity for the improvement of wine aroma (Smit *et al.*, 2003). Many species within the *Enterobacteriaceae*, common wort contaminants, are associated with the formation of volatile phenolic off-flavours. The ability to decarboxylate HCA's is present in *Klebsiella* (*K. aerogenes* and *K. oxytoca*), *Enterobacter* (*E. cloacae* and *E. aerogenes*) and *Hafnia* (*H. protea* and *H. alvei*) (Finkle *et al.*, 1962; Hashidoko and Tahara, 1998; Lindsay and Priest, 1975; Parry *et al.*, 1962). Among the beer contaminants, some acetic acid bacteria (e.g. *Acetobacter aceti*) and many lactic acid bacteria have been found to be able to decarboxylate pCA and FA (e.g. *Lactobacillus brevis*, *L. fermentum*, *L. plantarum*, *L. brevis*, *L. hilgardii*, *Pediococcus pentosaceus*, *P. damnosus* and *P. cerevisiae*) (Barthelmebs *et al.*, 2000; Chatonnet *et al.*, 1992; Chatonnet *et al.*, 1993; Chatonnet *et al.*, 1995; Gury *et al.*, 2004; van Beek *et al.*, 2000). Some *Lactobacilli* (like *L. plantarum* and *L. pastorianus*) are also able to reduce the vinyl derivatives to the corresponding ethyl derivatives similar to *Brettanomyces* spp. Some bacteria (e.g. *Pseudomonas* spp. and *Bacillus coagulans*) can grow on FA as a sole carbon source. Here, instead of a decarboxylation reaction (C1-cleavage), the initial step in the degradation of FA is a C2-cleavage resulting in the formation of vanillin or vanillic acid. These compounds are transformed to protocatechuic acid, which is incorporated in the tricarboxylic acid cycle via the β-ketoadipate pathway (Barghini *et al.*, 1998; Karmakar *et al.*, 2000; Narbad *et al.*, 1998). However, since these microorganisms are malt or sweet wort contaminants, which are eliminated quickly during fermentation, it is unlikely that they affect the volatile phenolic aroma profile of beer.

I.1.4. Formation of volatile phenols during wort fermentation and evolution during beer ageing

Information about control mechanisms for the formation of volatile phenols during wort fermentation is scarce. Kieniger *et al.* (1984) postulated that the fermentation temperature is an important parameter and stressed that the selection of the yeast strain has a significant influence on the development of phenols. Back *et al.* (2000) examined the formation of volatile phenols during the production of Weizenbier. The type of fermentation vessel, its shape and size had a considerable effect on the sensory qualities of Weizenbier. Beers from a main fermentation in a tun or a horizontal fermentor had higher levels of 4VG than beers from a vertical or cilindroconical tank. A raised bottle conditioning temperature or increased duration of warm storage promoted the phenolic flavour impression. Formation of 4VG was slightly less pronounced in fermentations of high original gravity. McMurrough *et al.* (1996) found that there are appreciable temperature dependent losses of 4VG during the storage of beer. 4VG is transformed into currently unidentified compounds. In wine, the decrease of 4VP and 4VG has been attributed to the slow acid-catalysed addition of ethanol yielding 4-(1-ethoxyethyl)-phenol and 4-(1-ethoxyethyl)-guaiacol (Dugelay *et al.*, 1995). Vinylphenols can also undergo a cycloaddition with anthocyanins yielding red-pigmented pyranoantocyanins (e.g. malvidin-3-*O*-glucoside-4-vinylguaiacol) in wine (Hakansson *et al.*, 2003; Fulcrand *et al.*, 1996; Pozo-Bayon *et al.*, 2004; Wang *et al.*, 2003). When storing wine on yeast lees, volatile phenols may also diminish by the adsorption to the yeast cell membrane (Chassagne *et al.*, 2005).

I.2. ARABINOXYLANS IN BARLEY, MALT, WORT AND BEER

HCA's are the precursors of volatile phenols in wort and beer. Most of the HCA's in wort are released from the malt grist during mashing either in free form or in AX-esterbound form. Because of the importance of AX polysaccharides as substrate molecules for cinnamoyl esterase enzymes, their structure and physicochemical properties, their enzymatic degradation and their occurrence in barley, malt, wort and beer will be discussed in more detail.

I.2.1. Structure of cereal cell walls

I.2.1.1. Morphology of the barley kernel

Cereal grains produce one-seeded fruit called a caryopsis, more commonly referred to as grain or kernel. The barley (*Hordeum vulgare* L.) kernel (figure I.4), like other cereal grains, contains carbohydrates, proteins, lipids, minerals, vitamins and other minor compounds.

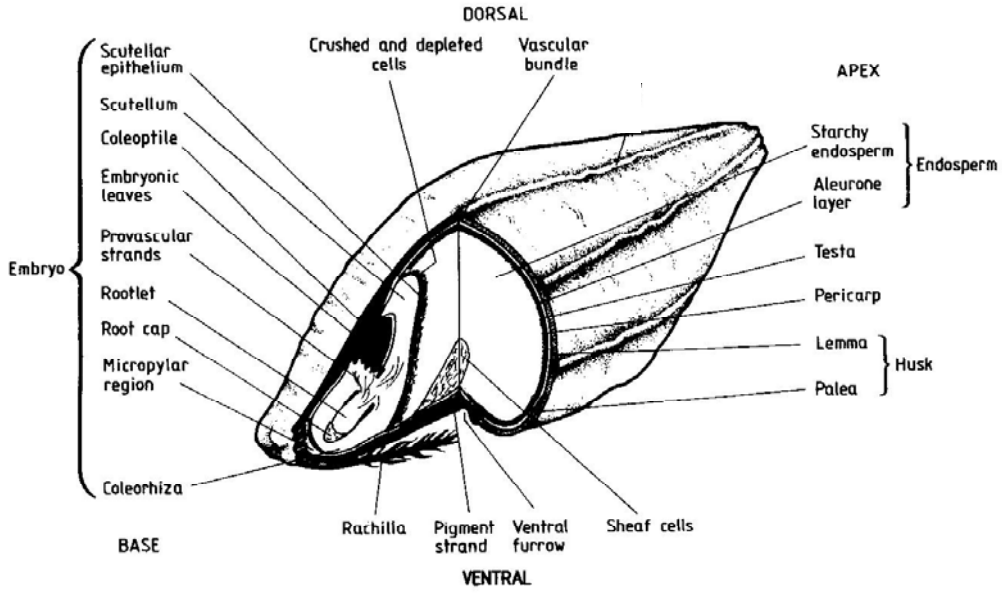


Figure I.4. *Structure of the barley* (Hordeum Vulgare *L.*) *kernel (Briggs, 1998)*

The outermost part of the grain, the husk, contains almost all lignin of the grain but hemicellulose and cellulose fibers are also present. Next is the pericarp (fruit coat), which has a chemical composition that resembles the husk except for the lack of lignin. The pericarp is closely attached to the testa (seed coat), which surrounds the endosperm. For brewers, the endosperm is the most valuable part of the grain. It consists of the aleurone layer and the starchy endosperm. The aleurone cells are cubical and arranged in a layer that is about three cells thick. It constitutes almost 5 % of the grain. The aleurone layer is built up mainly of AX and β-glucan. In barley, the aleurone cell walls contain about 70 % AX and 25 % β -glucan. The starchy endosperm occupies the bulk of the grain. Endosperm cell walls enclose the starch granules embedded in a protein matrix. In contrast to the cell walls of the aleurone layer, cell walls of the starchy endosperm contain mainly β–glucan (75 %) and only 20 % AX. The germ, which consists of scutellum and embryo, is rich in protein and fat. (Briggs, 1998; Fincher and Stone, 1986; Forrest *et al.*, 1977)

I.2.1.2. Primary and secondary cell walls in cereal grains

Cell walls of grain tissues perform a variety of physiological functions. During development and at maturity, they provide the skeletal framework of the tissues and the intercellular cohesive forces that maintain tissue integrity. They act as a conduit for movement of water and low-molecular-weight solutes through the grain. In addition, they provide a barrier to insect and microbial penetration into the cellular contents. At germination, the endosperm and aleurone cell walls are modified by hydrolytic enzymes as part of the process of mobilising the grain reserves (Briggs, 1998). The plant cell wall provides mechanical strength, maintains cell shape, controls cell expansion, regulates transport, provides protection, functions in signalling processing and stores food reserves (Bidlack *et al.*, 1992).

Primary cell walls are deposited during the elongation phase of cell development. Typically, primary cell walls are composed of cellulose microfibrils and an interpenetrating matrix of hemicelluloses, pectins and proteins. Approximately 90 % of the cell wall consists of carbohydrates, the remaining 10 % is protein. A proportion of these polysaccharides are hydrogen bonded to the surfaces of cellulose microfibrils and by virtue of their length, are able to interact with surfaces of more than one microfibril and so act as an adhesive between them. Within the matrix, there are also other, less well-defined, hydrogen-bonding possibilities between polysaccharides as well as ionic and salt interactions between polysaccharides and proteins (Iiyama *et al.*, 1994). Pectins provide cross-links and structural support to the cell wall whereas proteins can function either structurally (extensin) or enzymatically. The pectic content in cell walls of cereals such as wheat, maize and barley is relatively low. Proteins other than extensin found in the cell wall include enzymes such as hydrolases and oxidases needed for cell wall modification and lignification respectively during secondary growth (Bidlack *et al.*, 1992). Besides non-covalent interactions, also covalent cross-links occur. These will be discussed more in detail in paragraph I.3.1.

In contrast to the unlignified primary cell walls of the aleurone layer and the starchy endosperm, cell walls of the outer husk of cereal grains are further thickened by the deposition of a secondary wall layer that is rich in cellullose microfibrils arranged in parallel sheets and lignin. Secondary cell walls are derived from primary cell walls by thickening and inclusion of lignin in the cell wall matrix. Cellulose and hemicellulose appear to be more structurally organised in the secondary cell wall than in the primary cell wall (Bidlack *et al.*, 1992). Inclusion of lignin adds further rigidity to the matrix. Lignin is a copolymer of three

phenylpropanoid molecules: sinapyl, coniferyl and coumaryl alcohol. Lignin monomers originate from the phenylpropanoid pathway (paragraph I.3.2).

I.2.2. Structure and properties of arabinoxylans

I.2.2.1. Non-starch polysaccharides in cereal grains

Non-starch polysaccharides are carbohydrates, which are not held together by α-glycosidic linkages. The main non-starch polysaccharides in cereals are cellulose, β-glucan and AX (Egi *et al.*, 2004). Cellulose is a structural polysaccharide in cereal grains and is a major component in the husk and outer layers. Cellulose is composed of D-glucopyranose residues linked by β(1-4)-glycosidic bonds. Individual cellulose chains form a two-fold helix and hydrogen bonds between adjacent glucosyl residues lock the polysaccharide chain into a relatively inflexible conformation. Hydrogen bonds hold about 40 of these glycan chains together to form a microfibril. In wheat and barley endosperm cell walls, cellulose only accounts for a few percent of the total wall composition, whereas in the outer, lignified layer it may constitute up to 20 % of the cell wall (Fincher and Stone, 1986). The main component of the starchy endosperm cell walls of barley is β-glucan (70-75 %). It consists of long linear chains of glucose residues linked through both β(1-3) and β(1-4) linkages. The majority of barley β-glucan chains can be represented as a copolymer of cellulotriosyl and cellulotetraosyl units connected by β(1-3)-linkages while the rest consists of longer blocks of up to 20 consecutive β(1-4) glucosyl residues (Jin *et al.*, 2004). AX are the primary pentose-containing polysaccharides in barley. The use of the term “arabinoxylan” is relatively new and early works often refer to them as pentosans, pentose gums or hemicellulose (Egi *et al.*, 2004). In barley, 90 % of the water-soluble non-starch polysaccharides are β-glucan, while AX account for 10 % of the water-soluble non-starch material (Forrest *et al.*, 1977).

1.2.2.2. Structure and physico-chemical properties of arabinoxylans

AX are important structural polymers in the husk, pericarp, aleurone and endosperm in cereal grains. They consist of a backbone chain of β(1-4)-xylopyranose units in which xylose residues may be substituted with arabinose at *O*-2 and/or *O*-3 (Vietor *et al.*, 1992). Cinnamoyl groups can be esterified to the arabinofuranosyl residues at the *O*-5. The structure of a portion of a β(1-4)-xylan chain substituted at the *O*-3 atom of the xylosyl residue with a 5-*O*-*trans*-feruloyl-L-arabinofuranosyl substituent is represented in figure I.5. It seems that neither the type of substitution nor the distribution of the substitution patterns along the xylan backbone is the result of a random process. Biosynthetic mechanisms favour disubstitution

and the presence of clusters with high substitution patterns and low substitution patterns have been found (Gruppen *et al.*, 1992; Dervilly-Pinel *et al.*, 2004).

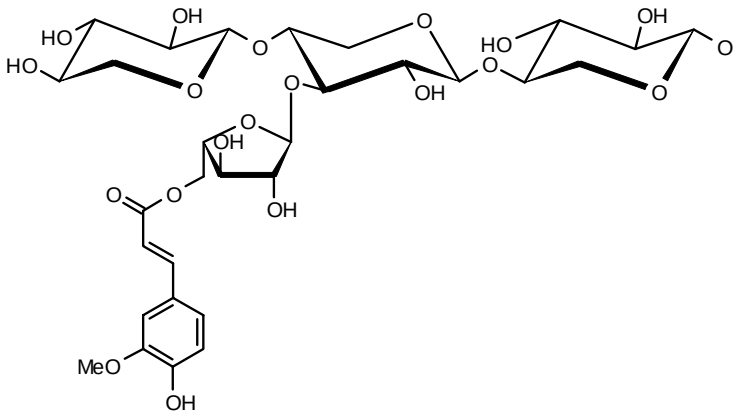


Figure I.5. *Structure of a portion of a β(1-4)-xylan chain substituted at the O-3 atom of the residue with a 5-O-trans-feruloyl-L-arabinofuranosyl substituent (Fincher and Stone, 1986)*

AX are high molecular weight, partially water-soluble carbohydrates (Fincher and Stone, 1986). Water-extractable AX (WEAX) can be separated from the water-insoluble fraction by an aqueous extraction. WEAX can be further separated by salting-out with ammonium sulphate or through precipitation using ethanol indicating that they are heterogeneous in structure. WEAX can form highly viscous solutions and can undergo oxidative gelation by cross-linking of esterified FA (paragraph I.3.1). Water-unextractable AX (WUAX) have the capability to absorb large amounts of water. The ratio of xylose to arabinose (Xyl/Ara) indicates the degree of branching. A higher proportion of arabinose indicates a more-branched polymer chain. Arabinoxylans exhibit a great deal of structural heterogeneity with respect to Xyl/Ara ratios, molecular size and arabinose substitution patterns. The physicochemical characteristics and functional properties of carbohydrate macromolecules depend on their degree of polymerisation and branching, and the distribution and type of arabinose linkages along the xylan backbone. The degree of arabinosylation increases the AX solubility in water. AX in unsubstituted form tend to aggregate into insoluble complexes that are stabilised by hydrogen bonds (Egi *et al.*, 2004). Schwarz *et al.* (1995) found the wort AX content to significantly correlate with wort viscosity. Inadequate degradation of AX during malting has been associated with problems in the brewery industry, such as diminished rate of wort filtration, haze formation in beer and reduced extraction efficiency (Han *et al.*, 1996; Sadosky *et al.*, 2002).

I.2.3. Enzymatic degradation of arabinoxylans

AX can be degraded by several enzymes (figure I.6). The endo-(1.4)-β-D-xylanhydrolases (EC 3.2.1.8), further referred to as endoxylanases, generate unsubstituted and substituted xylo-oligosaccharides. These oligomers are further degraded by β-D-xylosidases (EC 3.2.1.37) releasing β-D-xylose from the non-reducing end. The α-L-arabinofuranosidases (EC 3.2.1.55) release arabinose substituents from the main chain. Finally cinnamoyl esterases (EC 3.1.1.73) release FA and pCA esterified to the *O*-5 of arabinofuranosyl residues (Debyser *et al.*, 1998). The complete degradation of AX requires the concerted action of all AX-degrading enzymes.

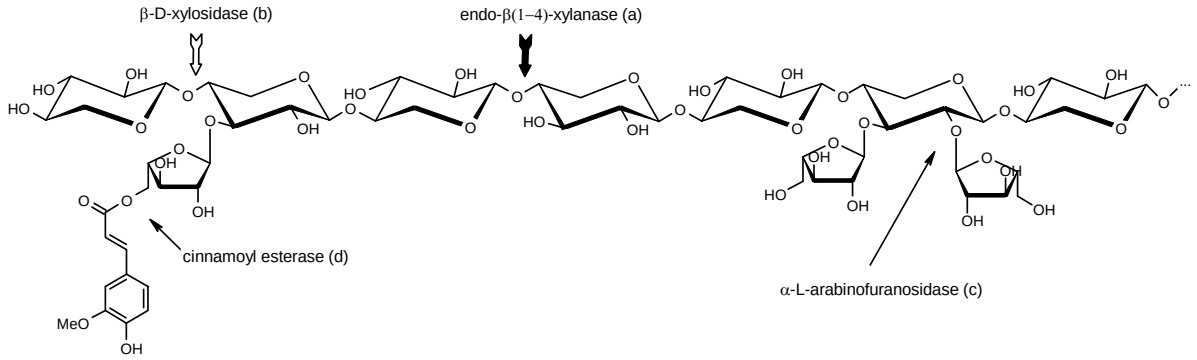


Figure I.6. Enzymes involved in arabinoxylan degradation (Debyser* et al.*, 1998)

(a) endoxylanase catalyses the hydrolysis of β(1-4)-xylosidic linkages within the xylan backbone releasing xylo-oligosaccharides of varying degree of polymerisation.

(b) β-D-xylosidase hydrolyses the β(1-4)-xylosidic linkages from the non-reducing end of the xylan backbone of xylo-oligosaccharides producing xylobiose and xylose

(c) α-L-arabinofuranosidase catalyses the hydrolysis of α(1-2) and α(1-3) arabinofuranosyl units

(d) cinnamoyl esterase hydrolyses esterified FA and pCA

I.2.3.1. Endoxylanase activity in barley

Endoxylanases catalyse the hydrolysis of β(1-4)-xylosidic linkages within the xylan backbone. Products are lower molecular weight arabinoxylan and (arabino)-xylo-oligosaccharides. Its action rapidly reduces the viscosity of WEAX (Preece and McDougall, 1958). Substitution of xylose at *O*-2 and *O*-3 appear to hinder degradation by endoxylanases (Vietor *et al.*, 1991). Consequently, polymeric wort AX are relatively highly substituted with arabinose. When isolated aleurone layers of barley are incubated with the phytohormone gibberellic acid, xylose and arabinose, both as free sugars and bound to larger oligosaccharides are released. Gibberellic acid, which originates in the growing embryo, induces the synthesis and release of endoxylanases (Dashek *et al.*, 1977). Preece and McDougall (1958) noted an increase of endoxylanase activity during germination. During

kilning, the activity substantially decreased, but the final activity in barley malt was significantly higher than the activity in raw barley. Sungurtas *et al.* (2004) reported that endoxylanase activity is very low in ungerminated barley and for the first two days of germination. Thereafter, it increases sharply until day 6 of germination. Slade *et al.* (1989) reported the purification and characterisation of three endoxylanases from extracts of germinating barley. They suggested that the enzymes are likely to function in the depolymerisation of cell wall AX during mobilisation of the starchy endosperm. All three endoxylanases were monomeric proteins of 41 kDa. They observed that the xylan hydrolase activity remained very low until 7-8 days after the initiation of germination. The purified xylan endohydrolases showed a requirement of at least three continuous xylosyl linkages in their substrate.

Caspers *et al.* (2001) have identified the major endoxylanase in the aleurone of germinating barley grain and showed that it is expressed as a precursor of 61.5 kDa with N- and C-terminal propeptides. It is synthesised as an inactive enzyme and only becomes active at the late stage of germination. A series of processing steps mediated by aleurone cysteine endoprotease yields a mature active enzyme of 34 kDa, which is secreted from isolated aleurone layers. The precursor is expressed at day 4 of germination while the active peptide is detected at day 10 of germination. All different forms of endoxylanase reported in barley probably arise from the same gene. This gene encodes the 61.5 kDa primary translation product. The enzyme of 41 kDa reported by Slade *et al.* (1989) is probably an intermediate form between the inactive 61.5 kDa precursor and the 34 kDa fully active endoxylanase enzyme. The 41 kDa intermediate form of the enzyme has low but detectable activity.

Glycoside hydrolases are grouped into families based on hydrophobic cluster analyses and on similarities in amino acid sequences in their catalytic site. The majority of the endoxylanases fall into families 10 and 11 (GH10 and GH11) of the classification scheme. All plant endoxylanases so far identified belong to family 10 (Simpson *et al.*, 2003). Both GH10 and GH11 endoxylanases are sterically hindered by arabinose substituents, but endoxylanases belonging to GH10 are able to cleave in more decorated regions being less hampered by the presence of substituents, while GH11 endoxylanases preferentially cleave in unsubstituted regions of the AX backbone. As a result, GH10 endoxylanases release shorter products than endoxylanases of family 11 (Kolenova *et al.*, 2006; Maslen *et al.*, 2007; Trogh *et al.*, 2005). Generally, a high degree of branching along the backbone is believed to impede degradation

by hindering the formation of the enzyme-substrate complex. On the other hand, AX with low degrees of substitution may associate, rendering the xylan chains less accessible to hydrolysis by enzymes.

Recently, it has been shown by Dornez *et al.* (2006) that the majority of wheat-kernel-associated endoxylanases consist of microbial endoxylanases and only a minority consists of native wheat endogenous endoxylanases. The distribution between microbial and native endoxylanases in malt is unknown. Although the malting of raw barley significantly reduces the total counts of viable moulds and yeasts, microcolonies of bacteria can still be detected on the surface of the malt kernel after kilning (Noots *et al.*, 1998). The latter together with the observation of Debyser *et al.* (1997c) that xylose solubilisation in worts prepared with 60 % barley malt and 40 % wheat was < 60 % of the xylose solubilisation in worts with 100 % barley malt, suggests that endoxylanase activity associated with barley malt is at least partly of microbial origin since endoxylanase inhibitor proteins are known to affect only microbial endoxylanases and not plant endoxylanases.

I.2.3.2. α-L-Arabinofuranosidase and β-D-xylosidase activity in barley

α-L-Arabinofuranosidase catalyses the hydrolysis of α(1,2) and α(1,3)-linked arabinofuranose units. Ferre *et al.* (2000) detected two α-L-arabinofuranosidase isoenzymes in germinating barley. One α-L-arabinofuranosidase was isolated from barley malt and displayed maximum activity at pH 4.2 and 60 °C. Arabinose was released both from monosubstituted and disubstituted xylo-oligosaccharides. β-D-Xylosidase catalyses the hydrolysis of β(1-4)-xylosidic linkages within xylo-oligosaccharides to yield xylose. β-D-Xylosidase has not yet been purified from barley, but its presence has been postulated by the observed release of monomeric xylose during mashing and the activity of barley and barley malt extracts towards artificial substrates. It has been partially purified from germinated wheat by Grant *et al.* (2003). Arabinofuranosidase and xylosidase are synthesised in response to gibberellic acid during barley grain germination (Dashek *et al.*, 1977). Sungurtas *et al.* (2004) reported that their activities in barley increased rapidly from day 1 through day 7 of germination. Xylosidase activities were always higher than arabinofuranosidase activities. Preece and McDougall (1958) noted that both xylosidase and arabinofuranosidase activities increased during germination and decreased during green malt kilning.

I.2.3.3. Cinnamoyl esterase activity in barley

Cinnamoyl esterases are able to hydrolyse the ester bond between HCA's and polysaccharides thereby releasing them from their bound state. Cinnamoyl esterases are also commonly referred to as cinnamoyl ester hydrolases, phenolic acid esterases, and feruloyl or *p*-coumaroyl esterases. They have been reported in raw barley and germinated barley (Sancho *et al.*, 1999) and have been partially purified from barley malt (Bartolomé *et al.*, 1996; Humberstone and Briggs, 2002; Sun *et al.*, 2005). Surface sterilisation showed that the activity was native to the barley grain rather than due to microbial contamination. The highest activity was detected after 2 days of germination although an overall downward trend was observed during germination. Barley and barley malt cinnamoyl esterases are active upon methylesters of pCA, FA and sinapic acid and upon the feruloylated oligosaccharide FAXX (5-*O*-feruloyl-α-L-arabinofuranosyl-(1-3)-β-D-xylopyranosyl-(1-4)-D-xylopyranose) (Bartolomé *et al.*, 1996; Sancho *et al.*, 1999). Cinnamoyl esterases are heat-sensitive (Sun *et al.*, 2005) and the activity has been reported to vary substantially among different barley varieties (Sancho *et al.*, 2001). Substantial lower levels of esterase activity in barley malt compared to barley were found and are due to its thermolability as well as to its decline during germination. Cinnamoyl esterases are optimally active at pH 7.5 and are only stable up to 30 °C (Humberstone and Briggs, 2002). Microbial cinnamoyl esterases have been classified in 4 classes (A, B, C and D) depending on their substrate specificity towards HCA methylesters and their ability to release diferulic acid cross-links (Faulds *et al.*, 2003). It is not known to which class barley cinnamoyl esterase belongs.

1.2.3.4. Synergy between cinnamoyl esterase and other AX-degrading enzymes

The decline of cinnamoyl esterase activity during germination suggests that the enzyme has a very early role in the modification of the cell walls of the starchy endosperm in barley facilitating the increased hydrolysis of both AX and β-glucan from endosperm cell walls by main-chain acting carbohydrases. Complete enzymatic hydrolysis of AX requires a battery of enzymes with both side-group cleaving and depolymerising main-chain acting enzyme activities. In general, plant cell wall degrading esterases demonstrate strong synergistic relationships with endo-acting main-chain degrading enzymes such as endoxylanases. In many cases, the synergy is reciprocal, where the action of one enzyme enhances the action of the other. Endoxylanase catalysed breakdown of polysaccharides generates low molecular weight fragments which are better suitable as esterase substrates, while the esterase releases phenolic groups facilitating the accessibility of the endoxylanase to the xylan backbone

(Mathew *et al.*, 2004). Synergistic action between microbial cinnamoyl esterases and other exogenous AX-degrading enzymes have been demonstrated on brewery spent grain (the water-insoluble part of the barley malt remaining after wort filtration). The release of FA from brewery spent grain by a cinnamoyl esterase of *Aspergillus niger* was increased more than 10-fold by the addition of a endoxylanase from *T. viride* (Bartolomé *et al.*, 1997). Two arabinofuranosidases from *Aspergillus kawachii* acted synergistically with endoxylanase in the degradation of AX and resulted in an increase in the amount of FA released by feruloyl esterases (Koseki *et al.*, 2003). In general, esterases have been found to be more active on WEAX than on WUAX. Most microbial esterases have been found incapable of releasing FA from brewery spent grain (i.e. WUAX of barley malt). Only in synergy with microbial AX-solubilising endoxylanases were they able to release FA (Faulds *et al.*, 2003). Native cinnamoyl esterase activity of barley malt was found to be enhanced by microbial *T. viride* endoxylanase. Barley malt extract containing only esterase activity hardly released any FA from brewery spent grain (Bartolomé *et al.*, 1996). Sancho *et al.* (2001) examined the release of FA from brewery spent grain by esterase-rich and endoxylanase-rich extracts from barley malt. The esterase-rich extract was able to release only very low amounts of FA from spent grain and wheat bran, indicating that it was not very active on WUAX. However, the presence of the endoxylanase-rich extract increased the release of FA significantly and demonstrated synergistic action between these two enzymes. They postulated that the endoxylanase activity is the limiting factor in the release of FA from brewery spent grain. Currently, little is known about the role of native cinnamoyl esterase and endoxylanase from barley malt in the release of FA during wort production and their activity on WEAX.

1.2.4. Occurrence and evolution of AX in barley, malt and wort

Henry *et al.* (1986) examined the genetic and environmental variation in AX in 17 varieties of barley grown at three different locations. AX levels were influenced by the variety and by the environment, which was the result of a complex interaction between rainfall, temperature, soil nutrients and other factors. The AX content varied from 4.4-7.8 %. Smaller grains contained relatively more AX indicating that the AX content may also correlate with the husk content. Slightly higher values were obtained by Lehtonen *et al.* (1987) who found AX levels in barley to range from 6.7-11%. They also reported the AX content of six-rowed barley to be significantly higher than that of two-rowed barley. Debyser *et al.* (1997a) determined total AX content of barley malt to range between 6.40 and 6.93 %, while the WEAX content ranged between 0.49 and 0.69 % indicating that the majority of AX in barley malt is water-

insoluble. The values are comparable to the values reported by Saulnier *et al.* (1995) for wheat: levels of 0.36-0.83 % and 5.53-7.79 % for water-extractable and total AX content were found, respectively. Ara/Xyl ratios of WEAX from barley malt ranged from 0.60 to 0.76. Methylation analysis showed a low proportion of *O*-3 monosubstituted xylose residues (3.4-10.7 %), a high proportion of disubstituted xylose residues (22.0-33.0 %) and the presence of *O*-2 monosubstituted xylose residues (5.1-12.8 %) the remaining part presenting unsubstituted xylose residues (Debyser *et al.*, 1997b; Dervilly *et al.*, 2002; Cyran *et al.*, 2002). Compared to barley, WEAX of wheat contained 67.4 % of unsubstituted, 19.5 % of mono-substituted at *O*-3 and 12.6 % of disubstituted xylose residues. In wheat AX, the amount of *O*-2 mono-substituted xylose residues is negligible (Cleemput *et al.*, 1995a). The molecular weight profiles of barley malt WEAX showed a peak at 38 kDa (Debyser *et al.*, 1997b). Compared to wheat AX, barley AX appear to be more structurally uniform where substituted regions are more clustered and separated by unsubstituted xylosyl residues (Vietor *et al.*, 1991). Concerning tissue composition, Barron *et al.* (2007) showed that peripheral layers of grains are mainly composed of AX but with differing structure. Compared to the starchy endosperm and the outer pericarp, both the hyaline layer and the aleurone layer consist of AX with low Ara/Xyl ratios (0.1 and 0.4, respectively compared to 0.8 for the starchy endosperm and 1.1 for the outer pericarp). These low ratios explain the high susceptibility of these tissues to endoxylanase degradation.

Both Sungurtas *et al.* (2004) and Dervilly *et al.* (2002) found WEAX levels in malt to be higher than those of the corresponding barley. This increase of WEAX after malting attests some AX solubilisation during the malting process. Li *et al.* (2005) observed a decrease of total AX content during germination while the content of WEAX increased. During kilning, only a small change in total and WEAX content was observed. Debyser *et al.* (1997b) obtained comparable levels of WEAX in barley and barley malt, but found a significant decrease in their average molecular weight indicating WEAX degradation during malting. During the brewing process, AX are both extracted (WEAX) and solubilised from WUAX by AX-hydrolases from the malt into the wort. Debyser *et al.* (1997a) examined the AX content in six commercial barley malts and their corresponding worts. During mashing, a significant amount of AX was solubilised by AX-degrading enzymes. A linear relationship between this solubilised AX and malt endoxylanase activity was found. Levels of monomeric xylose release were also related with β-D-xylosidase activity. However, only 9-35 % of total wort AX was solubilised indicating that most of the wort AX originated from WEAX in malt and

that endoxylanolytic levels of malts have only minor effect on the resulting total AX content of wort (Debyser *et al.*, 1997a). Also Li *et al.* (2005) observed that, during brewing, solubilisation of AX occurred, but only to a small extent. Debyser *et al.* (1998) examined the temperature dependence of AX-degrading enzyme activities during brewing. Endoxylanase and arabinofuranosidase activities remained constant until 50 °C, but rapidly decreased at higher temperatures. At 72 °C, the endoxylanase and arabinofuranosidase activities were almost completely lost. The xylosidase activity only slowly decreased at 63 °C. A similar temperature dependence of endoxylanase activity was found by Li *et al.* (2005). The latter also investigated the effect of mashing variables on the final AX content in wort. Mashing-in time, mash thickness, grist coarseness and stirring: all had a significant effect on the solubilisation and hydrolysis of AX during brewing. Increasing the mashing-in time and temperature led to elevated levels of AX. Although the endoxylanase enzyme was inactive at temperatures exceeding 70 °C, higher AX levels were found in isothermal mashes produced at 80 °C compared to 40 °C. This can be explained by the increased AX solubility at higher temperatures. During wort boiling, wort AX content decreased by 5-10 % because part of the AX were coprecipitated with proteins and polyphenols. The other steps in the beer production process did not have an effect on the levels of AX in beer (Debyser *et al.*, 1998). Final beer AX content has been found to range between 0.514 and 4.211 g/L and was several fold higher than the β-glucan content. It may account for up to 10 % of the total carbohydrate content in beer (Schwarz *et al.*, 1995).

I.3. HYDROXYCINNAMIC ACIDS IN BARLEY, MALT, WORT AND BEER

In this paragraph, the occurrence of HCA's in cereal cell walls, their origin and distribution in the cereal kernel and their evolution during malting and mashing will be reviewed.

I.3.1. Covalent cross-links between HCA's and cell wall polymers

In addition to non-covalent interactions between components of cereal cell walls, a number of mechanisms for covalent interactions exist. Geissmann and Neukom (1971) found that when feruloylated AX were treated with low concentrations of oxidising agents, they formed a gel. This led to the suggestion that FA residues could oxidatively dimerise and cross-link AX into

a firm gel. It was soon assumed that the same bridging action might be important in maintaining the structure of plant cell walls (Fry, 1979).

As bifunctional molecules with carboxylic and phenolic bonding sites, HCA's provide a pathway for intra- and intermolecular cross-linking between polysaccharides or between polysaccharides and lignin (Renger *et al.*, 2000). There are two potential methods for the covalent coupling of FA: photochemical cycloaddition leading to cyclodimers and radical-mediated dehydrogenative coupling catalysed by peroxidases leading to a range of dehydrodimers (Hatfield *et al.*, 1999). Peroxidase/H_2O_2 mediated oxidative coupling of FA esterified to AX in plant cell walls can form several FA dehydrodimers (figure I.7): 8-5', 8-O-4', 5-5' and 8-8' have been identified in insoluble dietary fibre of wheat, rye, barley, oat, maize, rice and millet (Mathew *et al.*, 2006; Ralph *et al.*, 1994). Also several FA trimers acids have been isolated from cereal grain namely 5-5/8-O-4-, 8-O-4/8-O-4- and 8-8/8-O-4-coupled dehydrotriferulic acid. These FA dehydrotrimers are presumed to cross-link polysaccharides like the dehydrodimers. Although it is theoretically possible, it is not known whether they are involved in the cross-linking of three distinct AX chains (Funk *et al.*, 2005; Rouau *et al.*, 2003).

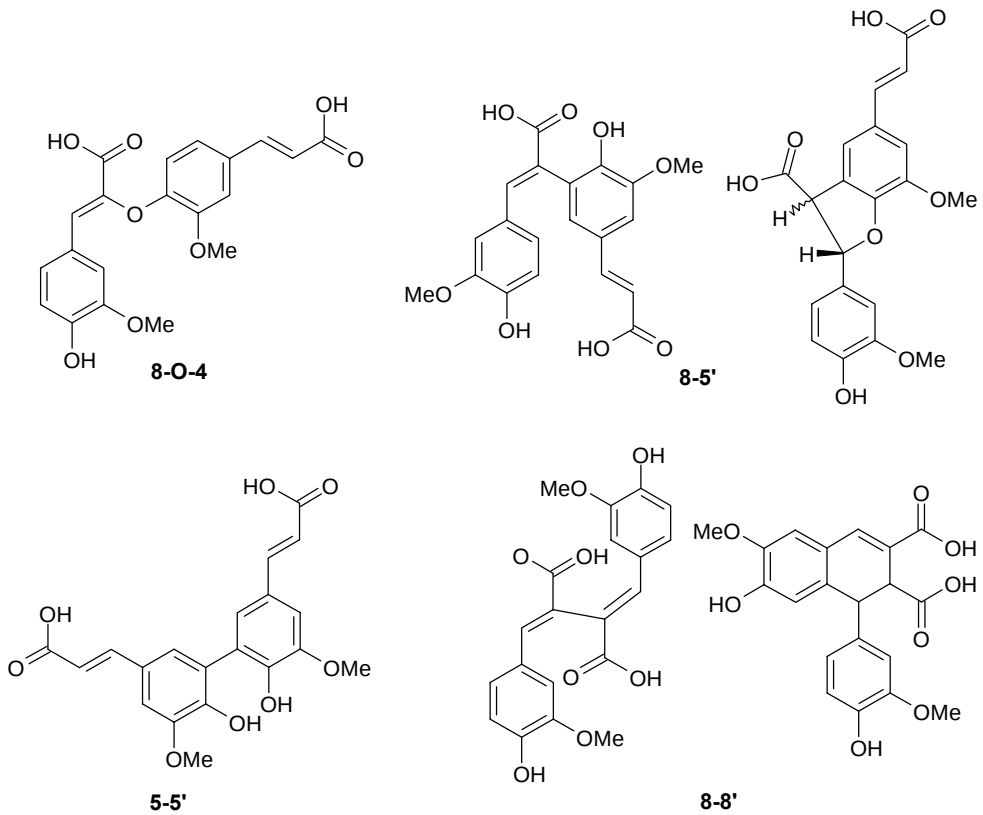


Figure I.7. *Structure of different dehydrodimers of FA (after Ralph* et al.*, 1994)*

HCA's can also dimerise by UV-induced cyclisation of the double bond of two propenoyl side chains between two HCA molecules yielding cyclobutane type dimers. Both head-to-head (truxillic acids) and head-to-tail (truxinic acids) cycloaddition products can be formed by this photodimerisation mechanism (Peyron *et al.*, 2002). Formation of head-to-tail and head-to-head homo- and hetero-cyclobutane type dimers of ester-linked pCA and FA have been demonstrated in the cell wall of grasses. Photodimerised HCA's have been reported in both lignified and unlignified walls, but not in primary walls. Radically coupled dehydrodiFA usually predominate over photochemically coupled cyclobutane derivatives in grasses (Funk *et al.*, 2005). Concerning the type of polymers involved in the cross-linking, it has been shown that HCA dimers are able to cross-link AX both in lignified and in unlignified cell walls by a dehydrodiferulic acid diester bridge. Both monomeric FA and dehydrodiferulic acids can enter the lignification process and become intimately bound up with the lignin complex (Ralph *et al.*, 1992). Jacquet *et al.* (1995) isolated dimers formed by radical coupling of FA and coniferyl alcohol indicating that ferulate esters are oxidatively copolymerised with lignin precursors. Apart from the cross-linking mediated by HCA's, also direct ester and ether linkages between polysaccharides and lignin have been found. Bifunctional FA can form covalent ester-ether bridges between polysaccharides and lignin or between two lignin fragments (Sun *et al.*, 2002).

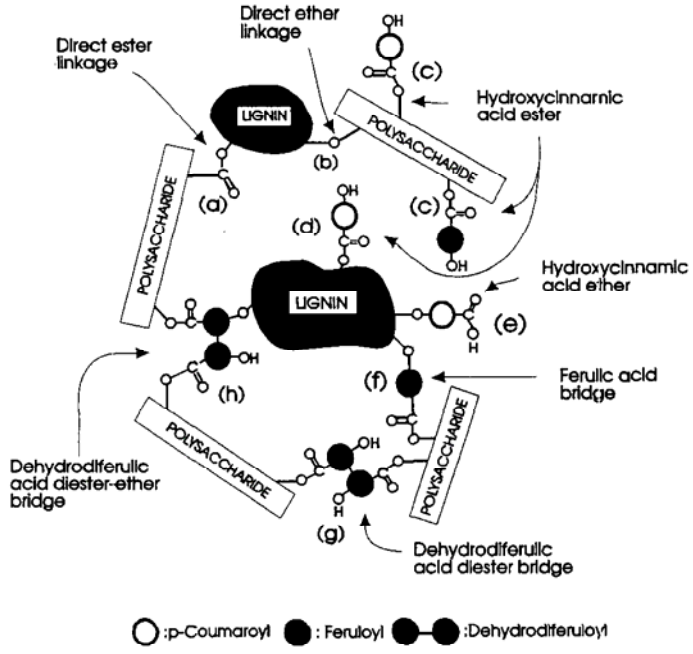


Figure I.8. *Covalent cross-links between polysaccharides and lignin in the plant cell wall: pCA and FA as cross-linking agents (Iiyama* et al.*, 1994)*

Dehydrodiferulic acid in diester linkage between two polysaccharides may also be etherified to lignin (Iiyama *et al.*, 1994). Possible interactions between polysaccharides, lignin and HCA's are shown in figure I.8. It has also been postulated that in the presence of tyrosine or cysteine, FA could link with proteins. However, currently no direct evidence for a protein-AX network has been found (Figueroa *et al.*, 1999). Recently, Piber and Koehler (2005) identified low levels of a dehydroFA-tyrosine cross-link in wheat and rye flours, which is likely to represent a covalent linkage between AX and cereal protein.

I.3.2. Origin and physiological role of HCA's in the plant cell wall

HCA's are phenylpropanoid compounds, which have a multiplicity of functions in plants like structural support, pigmentation, defence and signalling. Phenylpropanoids are derived from cinnamic acid (figure I.9).

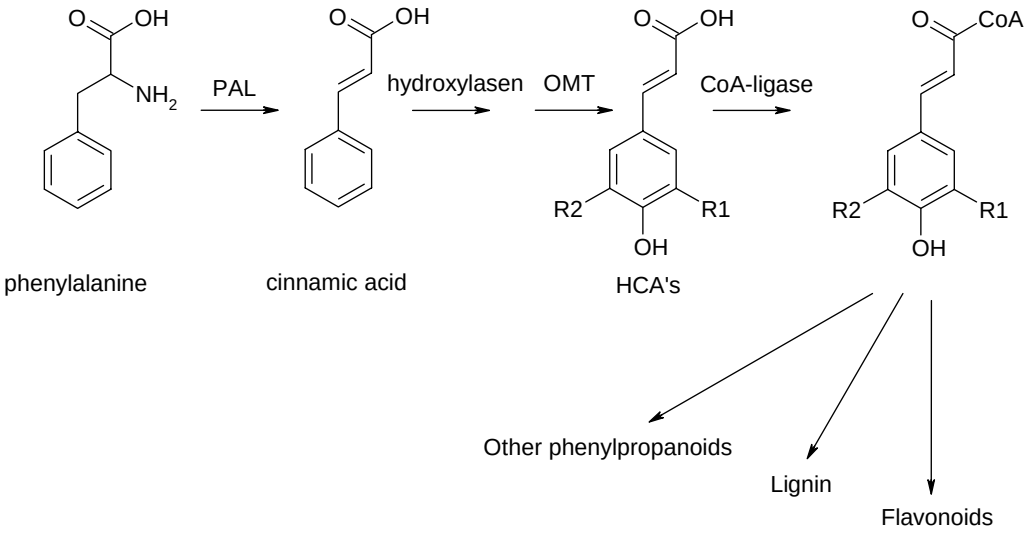


Figure I.9. *General phenylpropanoid metabolism in plants: deamination of phenylalanine by phenylalanine ammonia lyase (PAL) leads to the formation of cinnamic acid, which is further modified by the action of hydroxylases and O-methyl transferases (OMT) into hydroxycinnamic acids (HCA's). Activated HCA's are used in the biosynthesis of diverse aromatic compounds such as flavonoids and lignin (Douglas* et al.*, 1996)*

The enzyme phenylalanine ammonia lyase (PAL) catalyses the first metabolic step from primary metabolism into phenylpropanoid metabolism, which is the deamination of phenylalanine to produce cinnamic acid. The amino acid L-phenylalanine is itself derived from chorismate, the final product of the shikimate pathway. Cinnamic acid is further modified by the action of hydroxylases and *O*-methyltransferases (OMT) into HCA's. Most other phenylpropanoid compounds are derived from these HCA's. The enzyme 4-coumarate-CoA ligase catalyses the formation of CoA esters of HCA's. These activated intermediates are used in the biosynthesis of diverse compounds via specific branch pathways such as those

leading to flavonoid and lignin biosynthesis. Lignin monomers (*p*-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol) are thought to be derived from CoA esters of pCA, FA and SA via a two-step reduction process catalysed by cinnamoyl-CoA reductase and cinnamyl alcohol dehydrogenase (Douglas *et al.*, 1996; Brett *et al.*, 1999).

HCA's play a very important role in the life of the plant cell wall. Cross-linking among cell wall polymers can modify the mechanical properties of the cell wall affecting certain cell wall parameters such as extensibility. The presence of ester-linked monomeric and dimeric phenolics may also lead to reduced biodegradability of the cell wall polysaccharides preventing pathogen ingress (Faulds *et al.*, 1999). Sanchez *et al.* (1996) observed that HCA cross-linking decreased cell wall capacity for extension. Increased levels of FA have been found in plants acclimated to herbicide treatment and nutritional depletion (Rosazza *et al.*, 1995). They may also provide a physical barrier against invasive insects and microorganisms. Especially diferulate cross-links impede the potential enzymatic degradation of cell walls by fungal enzymes (Grabber *et al.*, 1998). In barley, FA levels have been found to increase in response to aphid infestation thereby reducing the severity of new infestations (Cabrera *et al.*, 1994). McKeehen *et al.* (1999) demonstrated that elevated levels of FA in wheat reduced the susceptibility of *Fusarium* head blight infection in wheat. Abdel-Aal *et al.* (2001) correlated differences in FA content in wheat with floret infestation levels with orange wheat blossom midge.

I.3.3. Distribution and occurrence of HCA's in cereal grains

Different levels of phenolic acids are found in different fractions of cereal grains. HCA's such as pCA, FA and SA are found both covalently attached to the plant cell wall and in soluble forms in the cytoplasm. Higher proportions of covalently bound FA are located mainly in the cereal brans, while pCA tends to be the main HCA in the stems of cereals (Faulds *et al.*, 1999; Sun *et al.*, 2002). Nordkvist *et al.* (1984) examined insoluble bound phenolic acids in 8 abraded fractions of the barley grain kernel (figure I.10). The outer layers, comprising the husk, pericarp, testa and aleurone cells, contained the highest concentrations of total phenolic acids, while their concentrations were considerably lower in the endosperm layers. The highest concentration of FA was found in fractions enriched with aleurone cells while the highest concentration of pCA was found in fractions containing high levels of

husks. This is consistent with the theory of pCA being primarily associated with lignin as the cell walls of the husks are considerably more lignified than those of the rest of the kernel.

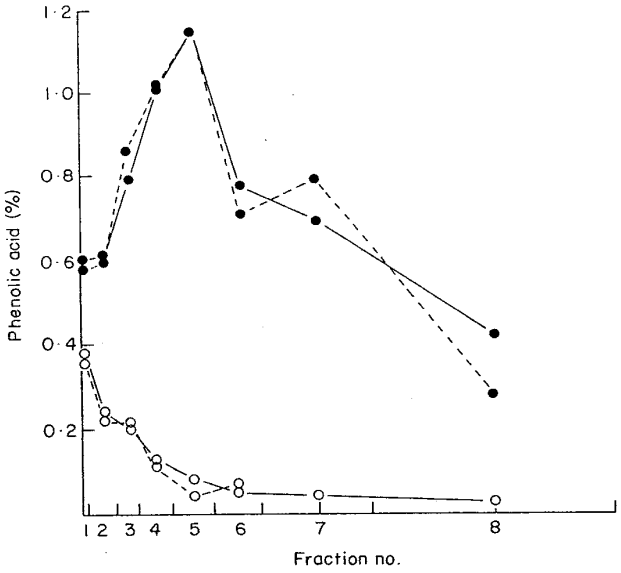


Figure I.10. *pCA (○) and FA (●) in 8 abraded fractions of the barley grain: fractions are numbered from the outside of the kernel to the inside of the kernel. Fraction 1 corresponds with the husk, fractions 3-5 correspond with the aleurone layer and fraction 8 corresponds with the endosperm (full line: HPLC detection, dotted line: GC detection) (Nordkvist* et al.*, 1984)*

About 75 % of total FA content in the barley kernel is bound to the outer layers (aleurone and husk) whereas 10 % is present in the endosperm (Bartolomé *et al.*, 1997). Also in wheat, Abdel-Aal *et al.* (2001) found FA concentrations to increase from the centre to the outer layers of the kernel. Phenolic acids are major phenylpropanoid components in cereals and can be separated into three categories: soluble free, soluble bound and insoluble bound. Adom *et al.* (2003) showed that more than 97 % of total FA in wheat was present in insoluble-bound forms, while only 0.2 % was present in free form. Yu *et al.* (2001) did not detect free FA in hot water extracts of barley indicating that also in barley HCA's are predominantly present in bound forms. In barley, wheat, rye and maize, FA has been found to be the most abundant phenolic acid, followed by pCA and SA. Levels of total alkali-extractable HCA's have been found to significantly differ between different cultivars of barley, wheat and rye (Abdel-Aal *et al.*, 2001; Adom *et al.*, 2003; Andreasen *et al.*, 2000; Bily *et al.*, 2004; Hernanz *et al.*, 2001). A genetic basis for FA levels in barley was inferred by a similar ranking of cultivars in two growing areas (Zupfer *et al.*, 1998). Phenolic acid concentrations were higher in six-rowed than in two-rowed barley (Yu *et al.*, 2001) and hulled varieties contained significantly

more phenolic acids than hull-less types (Holtekjolen *et al.*, 2006). In rye, differences between subsequent harvesting years were found (Andreasen *et al.*, 2000). Barron *et al.* (2007) showed that, compared to AX from the starchy endosperm, aleurone AX and hyaline AX have high FA/Ara molar ratios (7:100 and 10.7:100, respectively compared to 0.4:100 for the starchy endosperm). Interestingly, the tissues, which are the most susceptible to endoxylanase degradability (aleurone and hyaline layer) due to their low Ara/Xyl ratios (paragraph I.2.4), have the highest monomeric FA contents. This implicates that enzymatically solubilised AX are highly substituted with monomeric FA.

Total alkali-extractable FA levels in whole barley grain were found to range between 359-624 µg/g dry weight (Hernanz *et al.*, 2001; Zupfer *et al.*, 1998) while free FA levels in barley were only 2.51-3.87 µg/g dry weight (Zhao *et al.*, 2006). Among the dehydrodiFA identified in barley, 8-O-4' was the most abundant followed by the 5-5' and the 8-5' benzofuran form. FA dehydrodimers in barley are present at much lower concentration than the monomer (Hernanz *et al.*, 2001). Especially WEAX, both from barley and wheat, were found to contain only small amounts of FA dehydrodimers. Hartmann *et al.* (2005) showed that in WEAX from wheat, the percentage of FA residues involved in dimerisation was less than 1 % of the monomeric FA residues. Adams *et al.* (2005) found somewhat higher levels with monomeric FA residues being bound to 1 in 220 xylose residues in wheat WEAX while the quantity of dimeric linkages corresponded to about 1 linkage per 1400 backbone xylose residues. A study by Dervilly-Pinel *et al.* (2001) determined monomeric FA substituents in endosperm WEAX from wheat, barley, rye and triticale to range from 2-6 per 1000 xylose residues, while the content of FA dimers ranged from 0.3-18 per 100,000 xylose residues. In general, negative correlations were found between the amount of total dehydrodiFA and the solubility of AX indicating that dimers inhibit AX solubilisation (Beaugrand *et al.*, 2004).

1.3.4. Evolution of HCA's during malting and brewing

Most of the studies investigating HCA's in barley, barley malt, wort and beer have been conducted to examine their AO activity both in correlation to their potential for enhancing the flavour stability of beer as well as to the potential beneficial health effects attributed to them (Goupy *et al.*, 1999; Liyana *et al.*, 2005; Madhujith *et al.*, 2006; Mattila *et al.*, 2005; Nardini *et al.*, 2006; Pascoe *et al.*, 2003; Samaras *et al.*, 2005; Zhao *et al.*, 2006). Due to its phenolic nucleus and extended side chain conjugation, FA readily forms a resonance stabilised phenoxy radical, which accounts for its AO potential (Graf *et al.*, 1992). Traditionally, the

use of antioxidants in the brewing process has been minor due to the tendency to avoid additives and due to legislation. Raw materials for brewing like malt, other cereals and hops can be used as a natural source of antioxidants. Both free and bound HCA's have been studied for their potential for enhancing the flavour stability of beer. The AO activity due to bound HCA compounds has been found to be about 2-fold higher than the activity attributed to the free phenolic compounds (Maillard *et al.*, 1995). Zhao *et al.* (2006) suggested that the AO activity of beer could be increased through the screening of barley varieties with high levels of phenolic acids and correlated FA concentrations in barley extracts with their AO activity. Szwajgier *et al.* (2005b) examined the use of commercial enzyme preparations possessing cinnamoyl esterase activity to increase the free and water-soluble bound FA content in wort. However, most authors recognise the fact that an increased FA content in wort can cause unwanted phenolic off-flavours in beer. Hence, the proposed methods should, apart from pilsner beers, be used with the necessary precaution. Nardini *et al.* (2004) examined the HCA's content of pilsner beer after alkaline hydrolysis and showed that most phenolic acids in beer are present in bound form. They stated that for individuals who regularly consume beer, wine, coffee and tea, these beverages will likely be the major source of daily phenolic intake and that the phenolic acids are extensively absorbed by humans were they may reduce the risk of coronary heart diseases, cancer and ageing processes. Many studies have also been conducted on the FA content of brewers spent grain concerning validation of this waste product from the brewery industry. Mussatto *et al.* (2007) examined the release of FA and pCA by alkaline hydrolysis of brewers spent grain for the use of these phenolic acids in the food, cosmetic or pharmaceutical industry. Other authors examined the release of FA from brewery spent grain by microbial enzymes (Bartolomé *et al.*, 1997) and the subsequent conversion of FA to "natural" vanillin. The EEC legislation incorporated under the term "natural products" those produced from natural sources by living cells or their components including enzymes having GRAS (Generally Regarded As Safe) status. Beginning in 2000, Rhodia Inc. began marketing biosynthetic vanillin prepared by the action of microorganisms on FA extracted from rice bran. At $700/kg, this product, sold under the trademarked name Rhovanil Natural, is not cost-competitive with petrochemical vanillin, which sells for around $15/kg. However, unlike vanillin synthesised from lignin or guaiacol, it can be labelled as a natural flavouring agent. Currently, studies are conducted in an attempt to validate brewery spent grain in the same manner.

During the malting process (steeping, germination and kilning), the concentrations of FA, both in free form and monomeric or dimeric esterbound form, are known to increase (Dervilly *et al.*, 2002; Samaras *et al.*, 2005). The concentration of free FA in pale and lager malts was higher than in the corresponding raw barley. In contrast, no free FA was detected in black and chocolate malts due to the thermal decarboxylation of free FA to 4VG during the kilning or roasting process. During steeping, the total alkali-extractable FA content initially decreases slightly due to the leaching out of water-extractable free FA. During germination, the total and free FA content accumulated due to phenolic biosynthesis and hydrolysis of phenolic compounds bound to cell walls (Yang *et al.*, 2001). The use of gibberellic acid to induce germination promotes the cell expansion and favours the accumulation of wall-esterified FA and soluble phenolic compounds (Fry *et al.*, 1979). Elevated temperatures during steeping (22 °C *versus* 14 °C) and a decreased pH value (5.2 *versus* 7.4) of the steeping water caused significant increases in both free and water-soluble bound forms of FA (Szwajgier *et al.*, 2005a) in malt. During kilning, the content of bound HCA's first increased by about 130 % until 80 °C and then decreased by 20 % until 90 °C was reached in the kiln. The initial increase is possibly due to the changes in extractability caused by differences in tissue moisture content and not due to *de novo* synthesis. At higher temperatures, the decrease could be due to bound phenolic compounds being released during kilning by the action of temperature, the degradation of lignin or due to thermal degradation of FA (Maillard *et al.*, 1995). During kilning, also free FA levels initially increased upon heating to 80 °C with subsequent decreases. The increase in free phenolics early during kilning could be due to enzymatic release of bound phenolics and/or easier extractability due to changes in the matrix. Moreover, differences in the release of bound phenolics were found between different kilning regimes. Modification of the kilning regime could lead to a greater release of bound phenolics with consequent beneficial effects on flavour stability of beer and more generally on human health as stated by Inns *et al.* (2007). Woffenden *et al.* (2002) found the evolution of total bound phenolic acids during kilning, to parallel AO activity.

The cinnamic acid derivatives pCA, FA and SA have been reported in beer (Hayes *et al.*, 1987; Gorinstein *et al.*, 2000; McMurrough *et al.*, 1984; Nardini *et al.*, 2004). McMurrough *et al.* (1996) noted that malt contained only very small amounts of free phenolic acids but larger amounts were released on mashing the malt indicating cinnamoyl esterase activity during brewing. They also found that mashing-in at 65 °C significantly decreased the release of FA during wort production compared to mashing-in at 45 °C. Hence, there is a pronounced

dependency on temperature of the release of free FA during mashing. Narziβ *et al.* (1990) examined FA release at 20, 43, 60 and 85 °C and found it to be maximal at 43 °C. They found the optimal pH for free FA release to be 6.0. Back *et al.* (2000) found a higher 4VG content in beer after an intensified protein rest. This rest at 50 °C to ensure optimal protease activity leads to an increased release of FA. On the contrast, mash acidification led to a decrease in the phenolic flavour rating of the final beer. A proportional increase in the barley malt component of the throw compared to wheat malt, increased availability of FA. During wort boiling, Wackerbauer *et al.* (1982a) noted a small increase of FA by the addition of hop. In contrast, Pascoe *et al.* (2003) found no effect of hop addition on FA concentrations in wort. However, they used a supercritical CO_2 hop extract that probably contained very low levels of phenolic material. Little change occurred in the contents of phenolic acids on processing a lager wort through to the finished beer according to McMurrough *et al.* (1984). PVPP treatment of beer can cause a reduction of FA for up to 20 % (Papp *et al.*, 2001).

I.4. AIMS AND OUTLINE OF THIS STUDY

In recent years, volatile phenolic flavour compounds have been increasingly encountered in specialty beers. While some brewers wish for a clear phenolic note in (some of) their beers, others do not aim for their, sometimes overwhelming, presence in the taste pallet of beer. While the origin of volatile phenols in beer basically is known (arising from HCA's extracted from malt, which are subsequently decarboxylated during fermentation) not many means are known for the present-day maltster or brewer to optimise them in the final beer. Only few studies have been conducted concerning HCA's in relation to their flavour potential. The release of the precursors of volatile phenols during mashing has been mostly investigated in the light of their potential AO activity claimed to enhance beer flavour stability or even human health. While some of these results can be extrapolated to the optimisation of the volatile phenol content in beer, little is known of the dynamics behind the release of free HCA's during brewing, the interactive role of native barley enzymes underlying this release and the subsequent decarboxylation to the flavour-active compounds during wort fermentation. Also the causes of the temperature dependent decrease of 4VG during beer ageing need to be elucidated. The results presented in this study should lead to a better

understanding of the various mechanisms behind the formation of volatile phenols during the beer production process allowing for a better optimisation of their final concentration in specialty beers.

First (**chapter II**), an accurate method for the detection and quantification of HCA's and their volatile phenolic derivatives needs to be developed and validated. In **chapter III**, the method is used to perform an extensive survey on the occurrence of HCA's and volatile phenols in a range of beer styles. Tasting trials are performed to assess their contribution on the aroma of beer. HCA's and volatile phenols will be monitored throughout the beer production process to identify realistic control points for the final volatile phenol level in beer. The use of different barley malt varieties will be discussed in **chapter IV**. In **chapter V**, it is investigated whether, apart from the choice of a suitable barley malt variety, it is possible to influence the final wort HCA's content during brewhouse operations. The enzymatic decarboxylation of HCA's by yeast Pad1 activity is discussed in **chapter VI**. Finally, the decrease of 4VG during beer ageing will be examined in **chapter VII**.

CHAPTER II

DETERMINATION OF HYDROXYCINNAMIC ACIDS AND VOLATILE PHENOLS IN WORT AND BEER BY ISOCRATIC HPLC-ECD[1]

The development, optimisation and validation of a simple and rapid isocratic RP-HPLC method with amperometric detection for the simultaneous detection and quantification of hydroxycinnamic acids and their corresponding aroma-active volatile phenols in wort and beer is reported in this chapter. The technique gives good specificity and sensitivity, and can therefore be used for routine monitoring of hydroxycinnamic acids in wort and beer and the development of volatile phenolic flavour compounds during the beer production process.

[1] This chapter is based on the following publication: Vanbeneden, N., Delvaux, F., Delvaux, F.R. (2006). Determination of hydroxycinnamic acids and volatile phenols in wort and beer by isocratic high-performance liquid chromatography using electrochemical detection. *Journal of Chromatography A*, 1136, 237-242.

II.1. INTRODUCTION

There is extensive literature on the determination of HCA's for which predominately HPLC methods have been used (Floridi *et al.*, 2003; Garcia *et al.*, 2004; Hayes *et al.*, 1987; Jandera *et al.*, 2005; Lunte *et al.*, 1988). Gas chromatographic determinations were the methods of preference for the quantification of volatile phenols in alcoholic beverages (Callemien *et al.*, 2006; Carrillo *et al.*, 2006; Mejias *et al.*, 2003; Minuti *et al.*, 2006; Pollnitz *et al.*, 2000; Villareal *et al.*, 1986). However, analytical methods for the simultaneous determination of HCA's and their corresponding volatile phenols are very limited (Madigan *et al.*, 1994; McMurrough *et al.*, 1996). Moreover, the methods specified in these studies are not able to resolve all HCA's and volatile phenols present in wort and beer, or they have high detection limits for the analytes of interest. In this chapter, the development of a method for the simultaneous detection and quantification of HCA's and their derivatives in wort and beer is discussed. It is suitable for monitoring the occurrence of HCA's in wort and beer, the development of volatile phenolic flavour compounds during the beer production process and the evolution of these volatile phenols during beer ageing. The compounds of interest were the phenolic acids pCA and FA and their corresponding decarboxylation products (4VP and 4VG). SA was included in the study because it has been reported in beer (Wackerbauer *et al.*, 1982a) and may interfere with the quantification of FA because of their high similarity in structure. Possible reduction and oxidation products were included in the method to study chemical transformations during beer ageing (namely hydroferulic acid, 4EP, 4EG, vanillic acid, 4-hydroxybenzoic acid, 4-hydroxybenzaldehyde, vanillin, acetovanillon and vanillyl alcohol). The chemical structures of the analytes are represented in figure II.1.

Preference was given to high performance liquid chromatography (HPLC) using amperometric detection. HPLC coupled to electrochemical detection (HPLC-ECD) has become a widely accepted and valuable technique because of its high sensitivity as well as its superior selectivity to UV absorption for analytes that are electrochemically oxidisable (Kilmartin *et al.*, 2001). HCA's and their derivatives can be relatively easy electrochemically oxidised while forming quinoid forms by an electron transfer to the working electrode. Moreover, HPLC separation alleviates the need for lengthy sample preparation and/or derivatisation steps required for the gas chromatographic determination of non-volatile compounds.

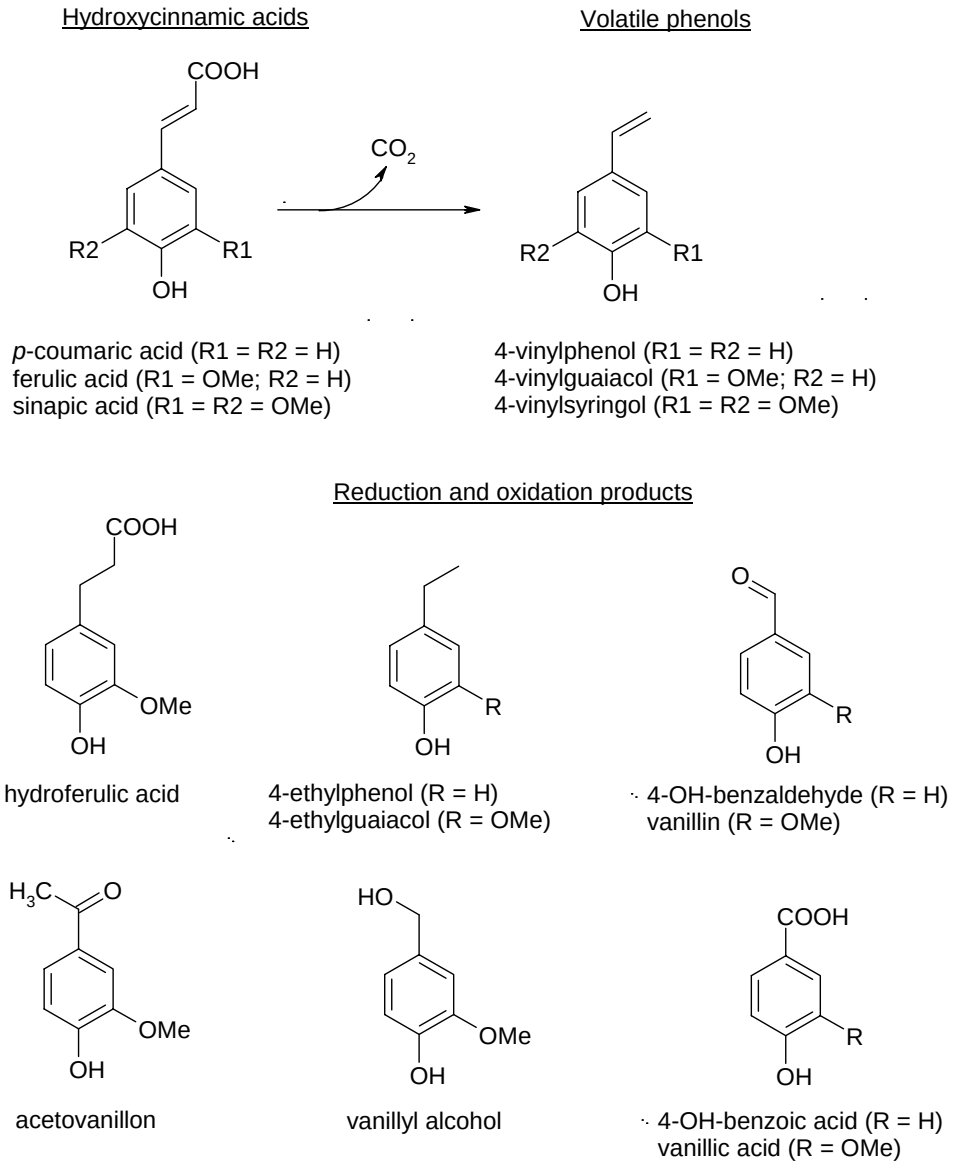


Figure II.1. *Structure of hydroxycinnamic acids, volatile phenols and their reduction and oxidation products*

II.2. MATERIALS AND METHODS

II.2.1. Chemicals and reagents

All analytes were of analytical grade. pCA, FA, SA, vanillic acid, 4EP, 4-hydroxybenzoic acid, acetovanillon, 4EG, 4VG, vanillyl alcohol, 4-hydroxybenzaldehyde and 4VP (10 % in propylene glycol) were obtained from Sigma-Aldrich (Bornem, Belgium). Vanillin and hydroferulic acid were obtained from UCB (Brussels, Belgium) and Apin Chemicals

(Abingdon, UK), respectively. For mobile phase preparation, analytical grade water type I (18.2 MΩ/cm - according to ASTM standards) was used. HPLC-grade methanol and *o*-phosphoric acid (85%) were purchased at AcrosOrganics (Geel, Belgium) and VWR (Leuven, Belgium), respectively. The mobile phase was degassed with helium prior to use.

II.2.2. Instrumentation and method optimisation

The Dionex (Sunyvale, CA, USA) DX500 chromatography system consisted of a Rheodyne (Rohnert Park, CA) model 9125 automatic sample injector (10 µL sample loop), a Dionex AS40 autosampler, GP40 gradient pump and ED40 electrochemical detector, which was operated in DC amperometric mode with a glassy carbon working electrode and an Ag/AgCl reference electrode. The output range was set at 100 nA. Chromatography was performed at room temperature. Separations were performed on a 250x4 mm Nucleosil 100-10 C18 column (Machery-Nagel, Düren, Germany) in conjunction with a 8x4 mm Nucleosil 100-10 C18 guard column. To optimise the working potential for amperometric detection, hydrodynamic voltammograms of the HCA's occurring in beer and their derivatives were constructed by repeated injections with the detector set at progressively higher electrode potentials over the range of +300 mV to +1300 mV. In the method finally adopted, the potential of the working electrode was set at +1200 mV *versus* Ag/AgCl. The optimal mobile phase composition was determined by gradually adjusting the polarity of the mobile phase changing its methanol content. The final mobile phase composition was $H_2O/CH_3OH/H_3PO_4$ (745/245/10; v/v) using a flow rate of 1.0 mL/min. Peak areas were analysed with the Chromeleon chromatography management system version 6.5 (Dionex). Sample components were detected and/or identified by coelution with added standards and by comparison of retention times from a mixture of reference compounds. Due to electrode surface fouling, the glassy carbon electrode was regenerated by daily polishing and sonication.

II.2.3. Sample preparation

Before injection, all samples were decarbonated by sonication for 10 min and filtered through 0.45 µm PVDF syringe filters (Alltech, Deerfield, IL, USA) into autosampler vials, which were frozen at –18 °C until analysis. All samples were protected from light during operation to minimise the photo-isomerisation reaction to which HCA's are susceptible. Alkaline hydrolysis of esterbound phenolic acids in wort and beer was performed according to Nardini *et al.* (2002).

II.2.4. Calibration curves and linearity

The calibration curves for the different analytes were constructed with five calibration standards (20, 10, 5, 1 and 0.05 ppm) diluted from a stock solution (100 ppm in 30 % methanol). Peak areas were plotted *versus* the theoretical concentration and calibration curves were obtained from least-squares regression analysis. The method linearity was evaluated by the square correlation coefficients (r^2) of the calibration curves.

II.2.5. Lower limit of quantification, precision and accuracy

Lower limits of quantification (LLOQ) were defined as the concentration of the analyte that produced a signal-to-noise ratio of ten. Five samples of three different concentrations (20, 10 and 1 ppm) diluted from a stock solution (100 ppm in 30 % methanol), were analysed at three different occasions and the intra- and inter-day precision and accuracy were calculated. Quality control samples were prepared independently from the calibration standards. The accuracy was determined as the mean of the measure relative to the theoretical value and is reported as a percentage (%). The precision is expressed as the intra- and inter-day relative standard deviation (RSD). Precision and accuracy were calculated at the LLOQ.

II.2.6. Matrix effects from wort and beer samples

Matrix interferences of wort and beer compounds were studied by spiking commercial wort and beer samples with increasing amounts of reference compounds (0.05, 1, 5, 10 and 20 ppm) in triplicate. Industrial wort samples (12 °P) were obtained from the CMBS pilot brewery. Beer samples were obtained from local grocery stores. The slopes of the curves thus obtained were compared with the corresponding slopes obtained in the calibration with standards and the concentrations of the analytes measured in the unspiked wort and beer samples were compared with the concentration calculated from the standard addition experiments.

II.2.7. Statistical analysis

Results of the analyses are expressed as the mean ± standard deviation. Differences between means were considered statistically significant when the *p*-value of the two-tailed Student's *t*-test was < 0.05.

II.3. RESULTS AND DISCUSSION

II.3.1. Optimisation of the analytical HPLC-ECD method

For quantification of analytes by electrochemical detection, the signal (i.e. current resulting from the oxidation of the analytes at the working electrode surface) has to be independent of the potential applied to the working electrode. The hydrodynamic voltammograms (i.e. plots showing the signal due to the oxidation of a compound in function of the applied potential), of the HCA's occurring in beer and their derivatives are shown in figure II.2. The term "hydrodynamic" refers to the samples of reference compounds being "in motion" (together with the mobile phase) as opposed to stationary (or cyclic) voltammetry.

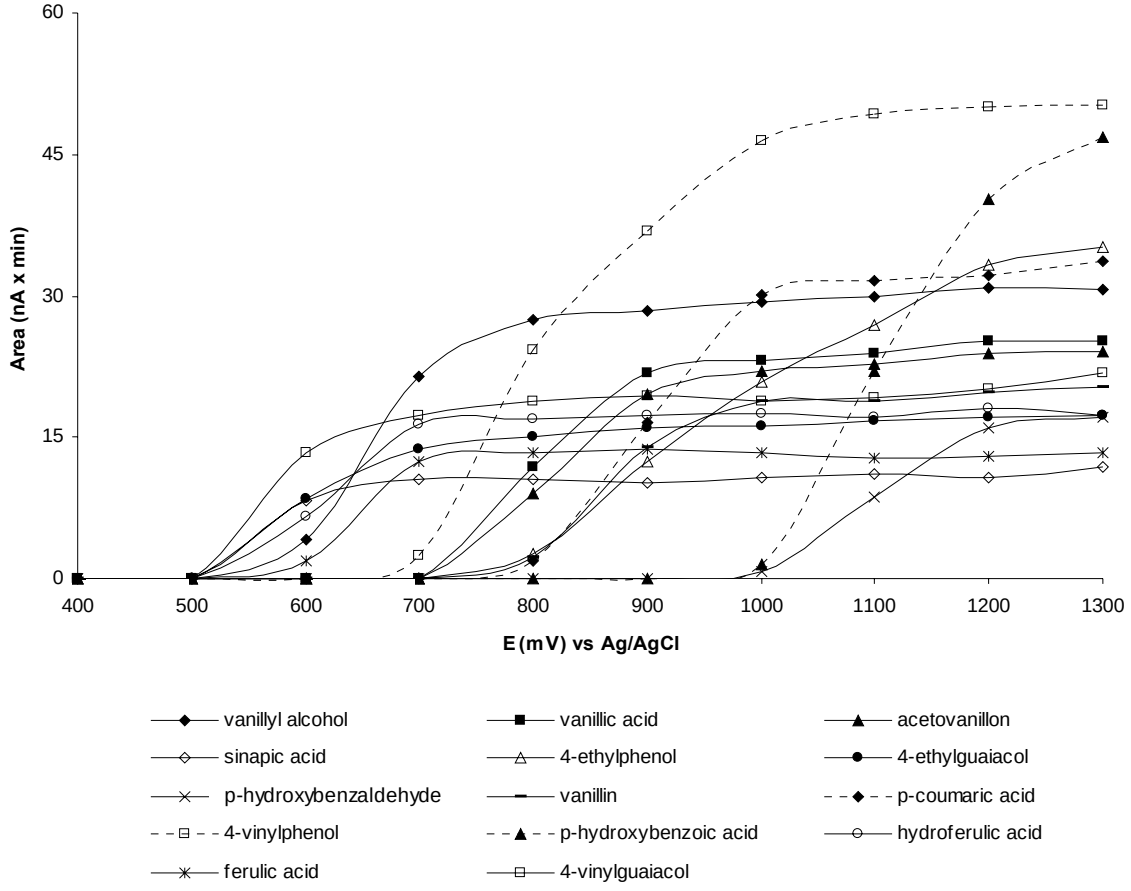


Figure II.2. *Hydrodynamic voltammograms of selected hydroxycinnamic acids and their derivatives*

It can be seen that individual selected compounds differ largely in their redox potentials. The redox potential of aromatic compounds is dependent on the extent of structural conjugation as well as the presence of electron-donating and electron-withdrawing substituents on the aromatic ring. The sequence of the redox potentials of the HCA's corresponds with their AO

potential (Jirovsky *et al.*, 2003). The more powerful reducing agent will have a less positive oxidation potential. At a detection potential of +1200 mV *versus* the Ag/AgCl reference electrode, a diffusion-limited current (i.e. a signal, which is independent of the applied potential) was achieved for all compounds of interest. Because of the low compatibility of gradient elution with amperometric detection at high operating potentials, isocratic elution had to be applied. Complete resolution of all compounds of a reference mixture on the C18 column was obtained by gradually adjusting the polarity of the mobile phase by changing its methanol content. Addition of *o*-phosphoric acid was used to improve the resolution by suppressing the dissociation of the weakly acidic phenolic compounds. The final composition of the mobile phase is described in the experimental section.

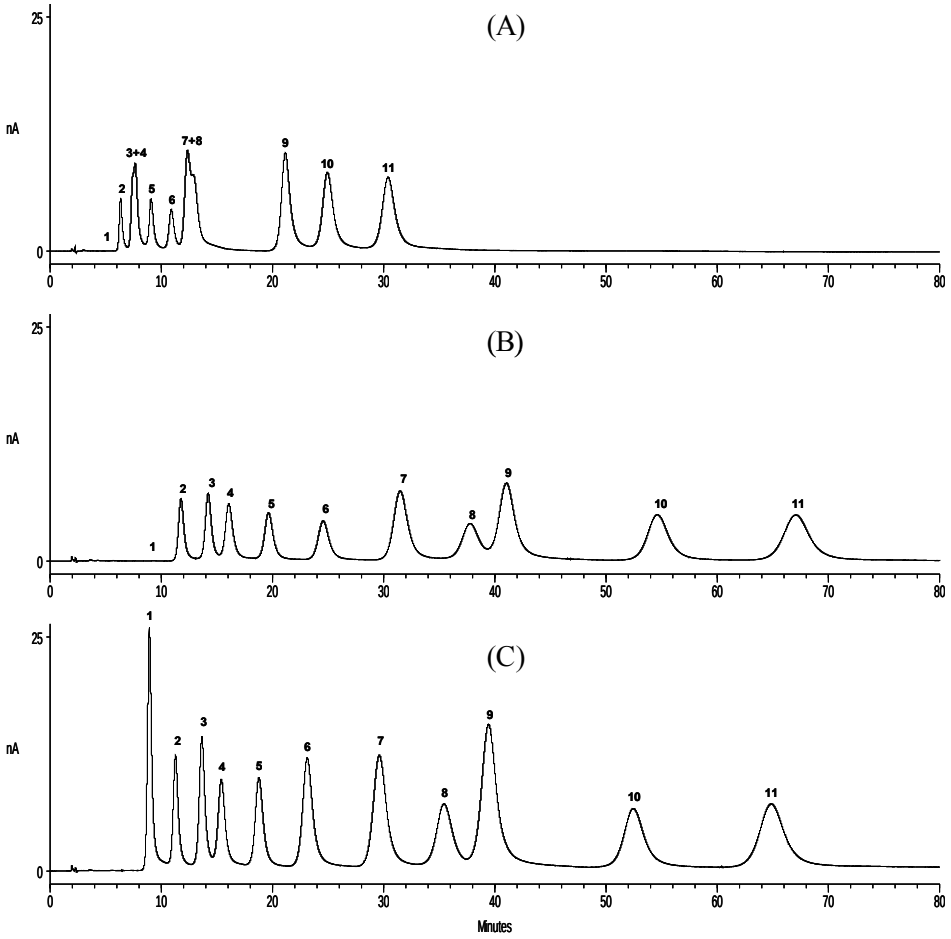


Figure II.3. *Effect of the mobile phase composition and the detection potential on the detection and quantification of simple phenolic compounds in wort and beer by HPLC-ECD: (A) H_2O:CH_3OH:H_3PO_4 640:350:10 v/v 900 mV; (B) H_2O:CH_3OH:H_3PO_4 745:245:10 v/v 900 mV; (C) H_2O:CH_3OH:H_3PO_4 745:245:10 v/v 1200 mV*

Figure II.3 illustrates the separation of a mixture of reference compounds obtained with the chromatographic conditions as described in this section.

II.3.2. Validation of the procedure

Good response linearity was obtained for all compounds studied up to 20 ppm with linear regression coefficients (r^2) of the calibration curves in a range of $0.9979 \leq r^2 \leq 0.9999$. The calculated calibration parameters and experimentally achieved lower limits of quantification (LLOQ) are summarised in table II.1. In previous studies, LLOQ's ranging between 0.01 and 0.07 ppm for pCA, between 0.01 and 0.20 ppm for FA and between 0.006 and 0.05 ppm for SA were obtained (Floridi *et al.*, 2003; Garcia *et al.*, 2004; Hayes *et al.*, 1987; McMurrough *et al.*, 1996; Pollnitz *et al.*, 2000). Concerning the volatile phenols, LLOQ's ranging from 0.005 to 1.244 ppm for 4VP, from 0.003 to 0.223 ppm for 4VG, from 0.017 to 0.021 ppm for 4EP and from 0.002 to 0.529 ppm for 4EG were obtained in previous published studies (Carrillo *et al.*, 2006; Diez *et al.*, 2004; Madigan *et al.*, 1994; Mejias *et al.*, 2003). For applications on wort and beer samples, the LLOQ's obtained with this method are sufficiently low compared with the flavour threshold of HCA's and volatile phenols in beer as reported by Meilgaard *et al.* (1975).

Table II.1. *Calibration parameters (slope, intercept and* r^2 *of the calibration curve) and lower limits of quantification (LLOQ)*

	LLOQ (ppm)	Intercept (nA.min)	Slope	r^2
p-coumaric acid	0.017	0.017	6.442	0.9989
ferulic acid	0.056	0.047	2.632	0.9995
sinapic acid	0.083	0.051	2.141	0.9997
4-vinylphenol	0.018	0.157	10.309	0.9984
4-ethylphenol	0.050	0.229	6.645	0.9999
4-vinylguaiacol	0.056	0.018	4.015	0.9996
4-ethylguaiacol	0.126	0.265	3.441	0.9979

The intra-day precision calculated from five replicate injections on the same day at 1 ppm was < 1.5% for each compound. The intra-day and inter-day precisions and accuracies for FA, the most abundant HCA in wort, and 4VG, the most abundant volatile phenol in beer, are summarised in table II.2. For the study of the matrix effects, one wort sample and three beer samples (i.e. pilsner beer, wheat beer and dark specialty beer) were used. The amount of HCA's and volatile phenols present in the unspiked samples as calculated with the calibration curves are expressed in table II.3. The results of the standard addition experiments for FA are summarised in table II.4. No significant differences could be found between the slope of the

calibration curve and the slopes of the standard addition curves obtained in wort and beer samples. Also, comparing the amount of FA calculated from the standard addition experiments with the amount of FA measured in the unspiked wort and beer samples, no significant differences could be found. However, routine analysis showed that with a few yeast strains, interference of an unknown fermentation metabolite with pCA can occur. This can sometimes impede the quantification of this HCA.

Table II.2. *Intra-day and inter-day accuracy and precision of reference samples diluted from a stock solution (100 ppm in 30 % methanol) for quantification of FA and 4VG at different concentrations (n = number of injections)*

Ferulic acid			
Concentration (ppm)	**Accuracy (%)**	**Precision (%)**	**n**
Intra-day			
20	100.4	0.51	5
10	99.9	0.86	5
1	100.8	0.67	5
0.056*	99.1	1.96	5
Inter-day			
20	100.3	1.06	15
10	99.9	1.47	15
1	100.3	0.90	15
4-Vinylguaiacol			
Concentration (ppm)	**Accuracy (%)**	**Precision (%)**	**n**
Intra-day			
20	98.7	0.59	5
10	102.0	0.29	5
1	103.7	0.65	5
0.056*	99.4	1.63	5
Inter-day			
20	99.8	1.24	15
10	99.5	1.40	15
1	103.9	1.80	15

*LLOQ of the method

Table II.3. *Amount (ppm) of hydroxycinnamic acids and volatile phenols present in unspiked wort and beer (calculated with the corresponding calibration curves) (ND: not detectable)*

	Wort	**Pilsner beer**	**Wheat beer**	**Dark specialty beer**
p-coumaric acid	1.479 ± 0.006	1.420 ± 0.021	ND	0.240 ± 0.003
ferulic acid	2.627 ± 0.009	2.225 ± 0.011	ND	0.390 ± 0.004
sinapic acid	0.128 ± 0.003	0.245 ± 0.003	0.504 ± 0.005	0.314 ± 0.004
4-vinylphenol	ND	0.045 ± 0.001	0.325 ± 0.003	0.309 ± 0.004
4-ethylphenol	ND	ND	ND	ND
4-vinylguaiacol	ND	0.139 ± 0.002	1.112 ± 0.017	0.587 ± 0.005
4-ethylguaiacol	ND	ND	ND	ND

Table II.4. *Standard addition experiments of FA in one wort sample and three beer samples*

	y = mx + b [(a)]	**FA (ppm)** [(b)]
Calibration curve	y = 2.632(±0.110).x + 0.047(±0.003)	-
Standard addition in wort	y = 2.625(±0.005).x + 6.889(±0.008)	2.624 ± 0.006
Standard addition in pilsner beer	y = 2.626(±0.004).x + 5.840(±0.051)	2.224 ± 0.020
Standard addition in wheat beer	y = 2.628(±0.009).x + 0.038(±0.023)	0.015 ± 0.009
Standard addition in dark specialty beer	y = 2.616(±0.026).x + 1.042(±0.061)	0.398 ± 0.024

[(a)] regression equation of the calibration curve and the standard addition curves of ferulic acid
[(b)] concentration of ferulic acid as calculated from the standard addition curves (=b/m)

II.3.3. Stability within work-up procedure

The main drawback of the described method is the occurrence of detector fouling caused by contamination of the electrode surface by oxidation products due to the high detector potential. This urges the need for regeneration of the detector surface by polishing. The number of samples, which can be run, depends on the kind of sample being injected. For samples of unboiled high gravity pilsner wort, a shorter program can be run since these samples do not contain volatile phenols. Injecting high gravity wort, a minimum of 25 samples can be analysed without loss of detector sensitivity. This corresponds to the need of regeneration every 24 h. For beer samples, between 25 (pilsner beers) and 15 (dark specialty beers) samples can be processed. This corresponds to the need of regeneration every 24 to 36 h. After abrading the electrode on alumina powder, the former response was recovered. To diminish the possibility of interferences, resulting from the use of high potentials, detector stability could be enhanced to several days by sample liquid-liquid extraction with ethyl acetate. However, this was regarded too time consuming compared to the daily polishing process.

II.4. CONCLUSION

A RP-HPLC-ECD method for the quantification of simple phenolic compounds, namely HCA's and their corresponding volatile phenols, in wort and beer is described. The method provides good overall limits of quantification for the phenolic coumpounds of interest. The method is also well suited for the analysis of bound phenolic compounds present in wort and beer after alkaline hydrolysis as described by Nardini *et al.* (2002).

CHAPTER III

OCCURRENCE OF HYDROXYCINNAMIC ACIDS AND VOLATILE PHENOLIC FLAVOUR COMPOUNDS IN BEER: A SURVEY [2]

In this chapter, an extensive survey of the occurrence of hydroxycinnamic acids and volatile phenols in a variety of beer styles is described. The contribution of 4-vinylguaiacol to the overall flavour perception of top-fermented specialty beers is shown. Significant differences in hydroxycinnamic acids (both free and esterbound) and volatile phenol content between different beers are observed.

[2] This chapter is based on the following publication: Vanbeneden, N., Gils, F., Delvaux, F., Delvaux, F.R. Formation of 4-vinyl and 4-ethyl derivatives from hydroxycinnamic acids: occurrence of volatile phenolic flavour compounds in beer and distribution of Pad1-activity among brewing yeasts. *Food Chemistry*, 107(1): 221-230.

III.1. INTRODUCTION

During brewing, FA, pCA and SA can be released as free acids by cinnamoyl esterase activity. These flavour-inactive phenolic acids, having a flavour threshold as high as 600 ppm (Meilgaard, 1975), are available for decarboxylation in the subsequent stages of the beer production process. Since probably not all of the AX-esterbound HCA's in wort are released by cinnamoyl esterase activity during brewing, an amount may remain bound to water-soluble AX. Information about the content of free *versus* bound phenolic acids in beer is scarce (Nardini *et al.*, 2004). The objectives of the experiments described in this chapter were to quantify both free and esterbound HCA's and their corresponding volatile phenols in a variety of beer styles and to determine the impact of volatile phenols on the aroma of different beer styles. An extensive survey of the occurrence of HCA's and volatile phenols in a variety of beer styles was conducted. Odour and flavour thresholds of 4VG were determined in a diverse range of beer styles to determine the contribution of 4VG to the overall flavour perception of top-fermented specialty beers.

III.2. MATERIALS AND METHODS

III.2.1. Materials

Pilsner beers, ale beers (EBC colour 17-50 according to European Brewing Convention standards), blond specialty beers (EBC colour 6-16), dark specialty beers (EBC colour 50-150), Belgian white beers, German Weizenbeers and beers produced by mixed or spontaneous fermentation (lambic beers, gueuze beers and sour red ales) were obtained from local grocery stores.

III.2.2. Determination of total alkali-extractable HCA's contents in beer

For the determination of the total alkali-extractable HCA's content in beer, esterbound HCA's were released from AX by alkaline hydrolysis. For this purpose, 5.0 mL beer was mixed with 5.0 mL 2 N NaOH (Riedel-de-Haen, Seelze, Germany) in pyrex tubes. NaOH solution was supplemented with 1 % ascorbic acid (Sigma-Aldrich, Bornem, Belgium) and 10 mM EDTA (Sigma-Aldrich, Bornem, Belgium) to prevent substrate oxidation according to the method described by Nardini *et al.* (2002). After flushing the test tubes with nitrogen,

the mixture was incubated on a rotary shaker. After 24 h, the reaction was stopped by adding 5.0 mL HCl 4 N (prepared from HCl 37 %; Fisher Chemicals, Zurich, Switzerland) and 300 mg NaCl. HCA's were extracted three times with 10.0 mL ethyl acetate (Acros Organics, Geel, Belgium). After vacuum evaporation of the combined ethyl acetate fractions to dryness at 35 °C, the HCA's were dissolved in 5.0 mL methanol prior to HPLC-ECD analysis. Validation of the hydrolysis and extraction procedure was performed by adding known aliquots (10 ppm) of the methyl esters of pCA, FA, SA (Oxford Chemicals, Hartlepool, UK) to pilsner beer. The recoveries were 93.57 ± 4.11 %, 97.06 ± 3.64 % and 94.21 ± 5.97 % (n=3) for pCA, FA and SA, respectively.

III.2.3. Quantification of HCA's and volatile phenols

Quantification of HCA's and volatile phenols in beer was performed by HPLC-ECD as described in chapter II.

III.2.4. Contribution of 4-vinylguaiacol to the aroma perception of beer

III.2.4.1. Determination of odour and flavour thresholds in water and beer

The odour and flavour thresholds of 4VG were determined in water and in beer by smelling (nasal) and tasting (taste and retro-nasal), respectively. Threshold determinations were performed according to the forced choice modification of the ascending method of limits test as described in Analytica-EBC (EBC Analytica, 1998, method 13.9). The test is used to determine the lowest concentration of an added substance that can be detected by odour or taste. Assessors receive 6 sets of 3 beer samples each consisting of two control and one test sample placed at random. The test sample is spiked with the test substance in increasing concentration (dilution factor 2). Individual best estimate values of threshold (BET) are found separately for each assessor as the geometric mean of the highest concentration missed and the next highest adjacent concentration. Group thresholds are derived from the individual values as the geometric mean of the individual best estimate thresholds by the following equation:

$$group\ BET = inv\log_{10}\left[\sum_{i=1}^{n}\left(\log_{10}\left(\sqrt{\min\times\max} = individual\ BET\right)\right)_i \Big/ n\right]$$

with
- *min: the highest concentration erratically assigned*
- *max: half of the highest concentration erratically assigned*
- *n: number of assessors*

Tasting panels had at least 20 members. The threshold for 4VG was determined in water, in a pilsner beer (original extract content (Eorig) 12.1 °P, real extract content (Er) 3.8 °P, ethanol content (Alc.) 5.08 % w/v, pH 4.45, colour 6.5 EBC, bitterness units 20.9 EBU, 0.139 ppm 4VG), a blond specialty beer (Eorig 17.2 °P, Er 4.9 °P, Alc. 8.32 % w/v, pH 4.17, 7.1 EBC, 31.9 EBU, 0.470 ppm 4VG) and a wheat beer (Eorig 12.0 °P, Er 4.1 °P, Alc. 5.20 % w/v, pH 4.61, 6.5 EBC, 11.8 EBU, 0.136 ppm 4VG). Beers were chosen because of low native 4VG concentration and as characteristic representatives within their respective beer style without having important off-flavours. Assessors were asked to describe the flavour perception of the spiked beer samples.

III.2.4.2. Recognition threshold of 4VG and effect on beer flavour appreciation

Sensory tests were carried out using a trained panel of 11 members. Each tasting trail, 1 blank wheat beer (Eorig 12.0 °P, Er 4.1 °P, Alc. 5.20 % w/v, pH 4.61, 6.5 EBC, 11.8 EBU, 0.136 ppm 4VG) or blond specialty beer (Eorig 17.2 °P, Er 4.9 °P, Alc. 8.32 % w/v, pH 4.17, 7.1 EBC, 31.9 EBU, 0.470 ppm 4VG) and 6 test samples with increasing 4VG concentration were presented. Assessors were asked to indicate the test sample, which they could discriminate from the blank sample by a clear identifiable increase in phenolic flavour intensity. They were asked to score each beer on a 0-9 scale.

III.2.5. Statistics

Results of beer analyses are expressed as the mean ± standard deviation. Differences between means (in µM) were considered statistically significant when the *p*-value of the two-tailed Student's *t*-test was < 0.05 (95% confidence level). The equality of variances (heteroscedasticity *versus* homoscedasticity) was tested with the two-tailed F-test. Whenever appropriate, paired analysis was conducted.

III.3. RESULTS AND DISCUSSION

III.3.1. Free HCA's and volatile phenols in commercial beers

Commercial beers belonging to a diverse range of beer styles (bottom-fermented pilsner beers, top-fermented ale beers, Belgian white beers, German Weizenbeers, blond specialty beers and dark specialty beers) were analysed on the content of free HCA's and corresponding volatile phenols. The results are summarised in table III.1. Significant differences in HCA's and volatile phenol content between different beers were observed. 4VP and 4VG concentrations ranged from 0.047 to 0.963 ppm and from 0.053 to 3.764 ppm respectively. In each beer, the content of 4VG was higher than the content of 4VP (figure III.1). A large variability between beers was observed in the extent to which FA and pCA were decarboxylated (5.4% to 98.6% and 3.8% to 91.1% for FA and pCA, respectively). In each beer, FA was decarboxylated to a greater extent than pCA.

Table III.1. *Free hydroxycinnamic acids and volatile phenols content in commercial beers (ppm)*

	pCA **x ± S.D.[a]** **(min-max)[b]**	**FA** **x ± S.D.[a]** **(min-max)[b]**	**SA** **x ± S.D.[a]** **(min-max)[b]**	**4VP** **x ± S.D.[a]** **(min-max)[b]**	**4VG** **x ± S.D.[a]** **(min-max)[b]**
pilsner beer (n=5)[c]	1.335 ± 0.501 (0.798-1.940)	1.998 ± 0.450 (1.317-2.426)	0.319 ± 0.082 (0.208-0.426)	0.044 ± 0.012 (0.036-0.065)	0.138 ± 0.037 (0.087-0.175)
ale beer (n=5)[c]	0.924 ± 0.169 (0.730-1.043)	1.155 ± 0.804 (0.408-2.355)	0.275 ± 0.077 (0.141-0.331)	0.308 ± 0.290 (0.041-0.705)	0.492 ± 0.482 (0.065-1.084)
Belgian white (n=14)[c]	0.906 ± 0.431 (0.348-1.668)	1.469 ± 0.428 (0.897-2.021)	0.393 ± 0.085 (0.283-0.516)	0.261 ± 0.139 (0.055-0.488)	0.733 ± 0.391 (0.136-1.519)
German Weizen (n=9)[c]	0.826 ± 0.418 (0.204-1.338)	0.814 ± 0.442 (0.354-1.774)	0.371 ± 0.150 (0.133-0.602)	0.374 ± 0.217 (0.046-0.846)	1.106 ± 0.570 (0.165-1.961)
blond specialty (n=16)[c]	1.057 ± 0.989 (0.129-2.770)	1.252 ± 1.055 (0.071-3.270)	0.370 ± 0.180 (0.155-0.775)	0.556 ± 0.220 (0.206-0.963)	1.381 ± 1.033 (0.470-3.764)
dark specialty (n=8)[c]	0.415 ± 0.163 (0.199-0.597)	1.071 ± 1.005 (0.094-3.198)	0.375 ± 0.243 (0.092-0.879)	0.207 ± 0.207 (0.047-0.663)	0.514 ± 0.427 (0.053-1.118)
TOTAL (n=57)[c]	**0.903 ± 0.597 (0.199-2.770)**	**1.268 ± 0.808 (0.071-3.270)**	**0.364 ± 0.152 (0.092-0.879)**	**0.339 ± 0.247 (0.047-0.963)**	**0.870 ± 0.758 (0.053-3.764)**

[a] *x ± S.D.: mean ± standard deviation*
[b] *(min-max): concentration range*
[c] *n: number of analysed beers from different brands*

4-Vinylsyringol, the decarboxylation product from SA, was not detected with the current method in any beer. Possibly, this is due to the inability of brewing yeasts to convert this HCA. However, it has been reported in aged lager beers where it can originate from the acid hydrolysis of glycosides or the thermal decarboxylation of SA (Callemien *et al.*, 2006). Tressl

et al. (1976) and Wackerbauer *et al.* (1982a) have also reported the presence of 4-vinylsyringol in Rauchbeers. Here, the fume used for the production of smoked malt can be the source of 4-vinylsyringol as it is a product of the pyrolysis of lignin (del Rio *et al.*, 2001).

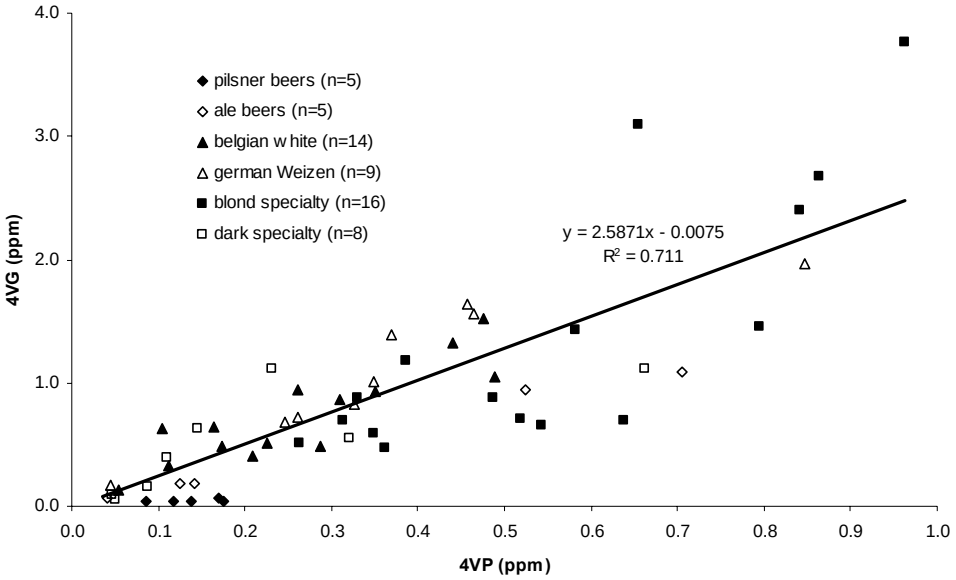


Figure III.1. *4VP* versus *4VG content in beers belonging to a diverse range of beer styles*

It can be seen from table III.1 that pilsner beers contain only small amounts of 4VP and 4VG (0.036-0.065 ppm and 0.087-0.175 ppm, respectively). Since these beers are fermented with bottom-fermenting yeasts, which do not posses Pad1 activity (Coghe *et al.*, 2004a; Perpete *et al.*, 2001), volatile phenols in pilsner beers probably arise only by thermal decarboxylation during high temperature treatments in the production process like wort boiling, transfer holding times and beer pasteurisation. In pilsner beers, only 3.8-5.8 % pCA and 6.9-9.6 % FA were decarboxylated. On the contrary, top-fermented beers like Weizenbeers, white beers and blond specialty beers contain significantly more 4VP and 4VG than bottom-fermented pilsner beers. The high levels of volatile phenols in top-fermented beers indicate a high incidence of the Pad1(+) phenotype among top-fermenting brewing yeasts. However, there seems to be a clear difference in their ability to convert HCA's. The highest quantities of volatile phenols were encountered in blond specialty beers. Although it is often thought that POF is typical only to wheat beers like Belgian white beers (made with unmalted wheat) and German Weizenbeers (made with malted wheat), clearly the presence of 4VP and 4VG is equally typical to blond specialty beers. In blond beers, between 20.3-91.1 % pCA and 23.2-98.6 % FA was decarboxylated to the corresponding volatile phenols. Strong blond beers contain significantly more volatile phenols than strong dark beers. This can be due to the inhibition of

the decarboxylase in dark worts during fermentation or due to the inhibition of the cinnamoyl esterase activity during brewing by compounds present in dark malts. The cinnamoyl esterase native to the malt may also be partially degraded during kilning and roasting at higher temperatures. However, since dark malts generally do not represent more than 5 percent of the total amount of raw materials, the latter can only be of minor importance. Further research with caramel, coloured and roasted malts has to be done to elucidate possible inhibition mechanisms.

III.3.2. Total alkali-extractable HCA's contents in commercial beers

Total alkali-extractable HCA's in beer equal the sum of free and AX-esterbound HCA's. The amounts of AX-esterbound HCA's can be calculated from the amounts of total alkali-extractable HCA's and the HCA's concentrations obtained from the analysis of the non-hydrolysed beer samples. Results are expressed in Table III.2. For each beer, the amount of free FA and pCA were compensated for the amount converted to 4VG and 4VP respectively to reflect the amount of free HCA's originally present in wort (i.e. available for decarboxylation). When taking the amount of corresponding volatile phenols into account, FA is the most abundant free phenolic acid in wort, followed by pCA and then SA. However, since FA is decarboxylated to a greater extent than pCA and the decarboxylation of SA is very limited, this initial profile can be considerably different in the corresponding beer. SA can even become the most abundant phenolic acid in beer when fermenting with a highly Pad1-active yeast strain. Taken into account the higher initial levels of FA in wort and the higher degree of decarboxylation of FA compared to other HCA's, 4VG was the most abundant volatile phenol in each beer (figure III.1). Due to the large variations in HCA's contents, no significant differences between pCA, FA or SA content can be found between the different beer styles.

From table III.2, it can be seen that considerable amounts of HCA's in beer occur in AX-esterbound form. Up to 76 % pCA, 95 % FA and 97 % SA may be present in bound form. In conjugated form, FA is the most abundant HCA followed by SA and then pCA. For each beer, the percentage of pCA occurring in free form *versus* the total pCA content (24.2-91.1 %) is higher than the percentage of FA in free form (4.9-28.7 %) and the percentage of SA in free form (3.2-22.5 %). Possibly this reflects the substrate specificity of the cinnamoyl esterase enzyme in malt. However, since the total amount of FA is considerably higher than

the total pCA and SA content, eventually, FA will be present in the highest absolute concentration in free form.

Table III.2. *Total free (compensated for 4VP and 4VG) and esterbound alkali-extractable hydroxycinnamic acids content (ppm) in commercial beers*

	TOTAL FREE HCA's			ESTERBOUND HCA's		
	pCA + 4VP	**FA + 4VG**	**SA**	**pCA**	**FA**	**SA**
	x ± S.D.[a]	**x ± S.D.[a]**	**x ± S.D.[a]**	**x ± S.D.[a]**	**x ± S.D.[a]**	**x ± S.D.[a]**
	(min-max)[b]	**(min-max)[b]**	**(min-max)[b]**	**(min-max)[b]**	**(min-max)[b]**	**(min-max)[b]**
pilsner beer (n=5)[c]	1.398 ± 0.517 (0.847-2.028)	2.176 ± 0.490 (1.429-2.605)	0.319 ± 0.082 (0.208-0.426)	0.585 ± 0.480 (0.155-1.270)	10.086 ± 2.264 (7.566-13.762)	1.963 ± 0.333 (1.578-2.436)
ale beer (n=5)[c]	1.328 ± 0.329 (1.054-1.694)	1.792 ± 0.481 (1.372-2.596)	0.275 ± 0.077 (0.141-0.331)	1.039 ± 0.160 (0.926-1.152)	11.749 ± 1.283 (9.769-13.108)	2.108 ± 0.111 (1.935-2.188)
Belgian white (n=14)[c]	1.286 ± 0.378 (0.903-2.061)	2.417 ± 0.476 (1.540-3.007)	0.393 ± 0.085 (0.283-0.538)	1.831 ± 1.100 (0.687-3.425)	10.157 ± 1.656 (7.250-13.005)	3.681 ± 0.950 (1.901-5.670)
German Weizen (n=9)[c]	1.359 ± 0.409 (0.681-1.974)	2.244 ± 0.666 (1.395-3.304)	0.371 ± 0.150 (0.133-0.602)	2.368 ± 0.977 (1.259-4.107)	9.803 ± 0.852 (8.285-11.133)	3.824 ± 0.781 (3.040-5.383)
blond specialty (n=16)[c]	1.990 ± 1.056 (1.113-3.477)	2.773 ± 1.222 (1.237-4.846)	0.332 ± 0.149 (0.155-0.554)	1.124 ± 0.231 (0.907-1.479)	13.644 ± 2.209 (9.475-16.337)	2.939 ± 1.007 (1.591-4.614)
dark specialty (n=8)[c]	0.791 ± 0.442 (0.421-1.434)	1.819 ± 0.945 (0.610-3.267)	0.293 ± 0.153 (0.092-0.470)	1.699 ± 0.469 (1.002-2.008)	11.952 ± 4.444 (9.069-20.834)	2.606 ± 1.429 (1.617-5.433)
TOTAL (n=57)[c]	**1.389 ± 0.638 (0.421-3.477)**	**2.332 ± 0.858 (0.610-4.846)**	**0.344 ± 0.125 (0.092-0.602)**	**1.620 ± 0.956 (0.155-4.107)**	**11.365 ± 2.620 (7.250-20.834)**	**3.077 ± 1.102 (1.578-5.670)**

[a] *x ± S.D.: mean ± standard deviation*

[b] *(min-max): concentration range*

[c] *n: number of analysed beers from different brands*

III.3.3. Volatile phenols in beers with mixed or spontaneous fermentation

In commercial beers (n=12) made with a mixed or spontaneous fermentation (lambic beers, gueuze beers and sour red ales), the vinylphenols 4VP and 4VG were only present in minor amounts (from not detectable levels up to 0.069 ppm and 0.258 ppm, respectively). Free pCA and FA could not be detected. However, ethylphenols were present in quantities from 0.063 to 0.730 ppm and from 0.427 to 3.605 ppm for 4EP and 4EG, respectively. These ethyl derivatives could not be detected in normal bottom or top-fermented beers. Chatonnet *et al.* (1992) detected these ethyl compounds in wine in which they have been typically ascribed to the presence of *Brettanomyces/Dekkera* spp. These yeasts may predominate at the end of the fermentation and maturation in wooden casks. The flavour of the ethyl derivatives is identical to that of the corresponding vinyl derivatives but their flavour threshold has been reported to be lower (Meilgaard, 1975). The formation of ethylphenols by *Brettanomyces/Dekkera* spp. in wine is attributed to the action of two sequential enzymes (Chatonnet *et al.*, 1992; Edlin *et al.*, 1995). First, the HCA's are decarboxylated to their vinyl derivatives by an enzyme

analogue to the Pad1 enzyme of *Saccharomyces cerevisiae*. The vinyl derivative is then reduced to the corresponding ethylphenol by vinylphenol reductase activity of *Dekkera/Brettanomyces* spp. *Saccharomyces cerevisiae* yeast strains are not able to execute this reductive step.

III.3.4. Contribution of 4-vinylguaiacol to the aroma perception of beer

III.3.4.1. Determination of odour and flavour thresholds in water and beer

The odour and flavour threshold of 4VG, the most important volatile phenol in beer, were determined in water and in beers belonging to different beer styles (pilsner beer, blond specialty beer and Belgian white beer). Odour and flavour thresholds of 4VP could not be determined due to the lack of a food grade standard. The values in water indicate the *absolute threshold* or the *stimulus threshold*, i.e. the lowest concentration by which 4VG can be detected. The values for the different beer styles represent the *thresholds of difference* as defined by Meilgaard *et al.* (1975) and Brown *et al.* (1978). This is the smallest difference in concentration, which can be detected as a just noticeable difference. The amount of 4VG pre-existing in beer is hereby ignored. Although threshold values may differ between beers belonging to the same beer style, the values offer important information on the overall flavour impact of 4VG. *Thresholds of difference* give a strong indication of the minimum level of change in concentration, which needs to be accomplished to achieve a perceivable change in the aroma perception of the beer. These values are important parameters for brewers wishing to change the phenolic aroma perception of their beer by changing the 4VG concentration. The odour and flavour threshold of 4VG in water, pilsner beer, wheat beer and blond specialty beer are indicated in table III.3.

Table III.3. *Odour and flavour threshold of 4VG in water and various beer media*

	Odour threshold (ppb)	**Flavour threshold (ppb)**
Water	88	< 20
Pilsner beer	294	125
Wheat beer	400	200
Blond specialty beer	516	367

Odour thresholds were higher than the corresponding flavour thresholds because of the limited volatility of 4VG and because of the possible dominance of other aroma-active compounds (e.g. esters and sulphur compounds) in the odour perception of beer. Although volatile phenol levels in pilsner beers were low compared to other beer styles, 4VG concentrations of three analysed pilsner beers (139, 170 and 175 ppb 4VG) exceeded the

flavour threshold of 4VG in pilsner beer (125 ppb). All strong blond beers had 4VG levels above the flavour threshold in blond specialty beer (up to 9 times the threshold) and all but one Weizen and one white beer, contained levels above the flavour threshold of 4VG in wheat beer. Flavour descriptions of 4VG in beer could be grouped in three major categories: (1) medicinal aroma (with flavour descriptors such as "dentist", "solvent", "detergent", "pharmaceutical" and "astringent") (2) spicy aroma (including "clove", "curry" and "nut meg") and (3) smoked aroma (including "BBQ", "roasted" and "rum").

III.3.4.2. Recognition threshold of 4VG and effect on beer flavour appreciation

The *threshold of difference* represents the smallest difference in concentration, which can be detected. This does not necessarily mean that, at this concentration, the phenolic aroma of 4VG can be recognised as such. This value is represented by the *recognition threshold* (i.e. lowest concentration permitting identification) and was determined to be 400 ppb and 760 ppb for the wheat beer and the blond specialty beer, respectively. For the two beers, the recognition threshold was twice as high as the flavour threshold. The effect of the supplementation of 4VG on the overall flavour appreciation of the wheat beer and the blond specialty beer are represented in figure III.2. Significant differences in total scores ($p < 0.05$ based on paired *t*-tests) are indicated.

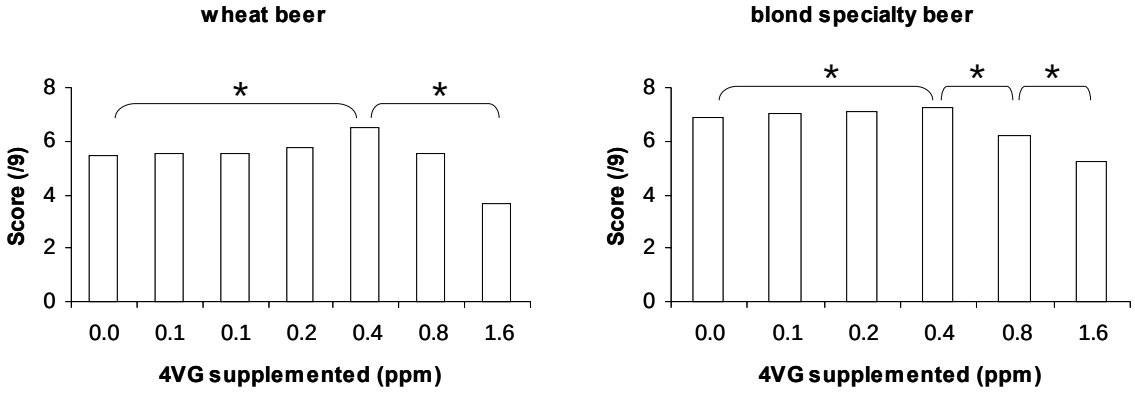


Figure III.2. *Effect of 4VG supplementation on the overall flavour appreciation of a wheat beer (left) and a blond specialty beer (right): beer samples were scored on a 0-9 scale. Significant differences (p< 0.05; based on paired comparison tests for each assessor) are indicated with an asterix*

At levels below the flavour threshold (0.2 ppm and 0.4 ppm for the wheat beer and the blond specialty beer, respectively), no significant effect of the 4VG concentration on the overall flavour appreciation could be found. Increasing the 4VG concentration to the recognition threshold had a remarked positive effect on the overall score of the wheat beer indicating that a perceivable phenolic flavour in this type of beer is appreciated. However, further increasing

the 4VG concentration above the recognition threshold had a remarked negative effect on the wheat beer flavour appreciation. Flavour descriptors changed from those perceived as positive (spicy, clove-like,…) to those perceived as negative (medicinal, astringent, pharmaceutical,…). When the 4VG concentration of the blond beer was increased to its flavour threshold, a consumer panel could discriminate the test sample from the blank sample. Although the phenolic 4VG flavour was not identified as such, the test sample received a higher overall score than the blank sample. The taste palate of the test sample was assigned "less complex" and "more balanced" than that of the blank sample. Both negative (solvent-like, acetaldehyde, sulfury, astringency) and positive (fruitiness, bitterness, hoppiness) flavour intensities decreased, but overall, the mouth fullness increased. However, when the 4VG concentration in the blond specialty beer was increased to the recognition threshold, the overall score significantly declined indicating that the presence of a perceivable phenolic flavour in this blond beer was generally not appreciated. It is also important to note that no flavour compound should be evaluated on facts and figures alone. In the perception of the phenolic flavour of beer, the appropriate balance between volatile phenols and esters is particularly important. The importance of balance phenolic *versus* fruity/estery will be highlighted in the results from the tasting trials of pilot-scale brewed beers in chapter VI.

III.4. CONCLUSION

In this chapter, an extensive survey of the occurrence of HCA's and volatile phenols in a variety of beer styles is presented. Odour and flavour thresholds of 4VG were determined in a diverse range of beer styles confirming the contribution of 4VG to the overall flavour perception of many top-fermented specialty beers (especially blond specialty beers and wheat beers). Significant differences in HCA's (both free and esterbound) and volatile phenol concentrations between different beers were observed. Differences in volatile phenol concentrations between bottom and top-fermented beers can possibly be explained by the relative importance of thermal *versus* enzymatic decarboxylation. The observed differences between top-fermented beers suggest a high incidence, but also large variability, of Pad1(+) phenotype among top-fermenting brewing yeast strains. The large variability in HCA's content between different beers otherwise having likewise properties (original extract

content, ethanol content,...) suggests that the release of HCA's during mashing may be influenced by various mashing processes and parameters. Analysis of total alkali-extractable phenolic acids showed that considerable amounts of HCA's in beer still occur in AX-esterbound form. Optimising this precursor release during mashing may be a means of controlling final volatile phenol levels in beer. The various hypotheses raised in this chapter will be further examined in following chapters.

CHAPTER IV

VARIABILITY IN THE RELEASE OF FREE AND ESTERBOUND HYDROXYCINNAMIC ACIDS FROM DIVERSE MALTED BARLEY (*HORDEUM VULGARE* L.) CULTIVARS DURING WORT PRODUCTION[3]

In this chapter, the variability in the release of free and esterbound hydroxycinnamic acids from nine malted barley varieties during wort production was investtigated. A large variability between different barley malts and their corresponding worts was observed. The release of ferulic acid during mashing did not only depend on the cinnamoyl esterase activity of malt, but also on the amount of esterbound ferulic acid initially present in the wort and on the endoxylanase activity.

3 This chapter is based on the following publication: Vanbeneden, N., Gils, F., Delvaux, F., Delvaux, F.R. Variability in the release of free and bound hydroxycinnamic acids from diverse malted barley (Hordeum vulgare L.) cultivars during wort production. *Journal of Agricultural and Food Chemistry*, *In Press.*

IV.1. INTRODUCTION

In the previous chapter, significant differences in HCA's concentrations (both free and esterbound) between different beers were observed. This large variability in HCA's contents between different beers otherwise having likewise properties (original extract content, ethanol content,...) suggests that the release of HCA's during mashing may be influenced by various mashing processes and parameters. Also the use of different kinds of raw materials may affect the phenolic acid content in wort. Optimising this precursor release during mashing may be a means of controlling final volatile phenol levels in beer. The objectives of the experiments described in this chapter were to investigate the variability in the release of free and esterbound HCA's between different malted barley (*Hordeum vulgare* L.) varieties, and their distribution between malt, wort and spent grain. Malt parameters that explain the observed variability in HCA's concentrations in worts from different barley malt varieties will be identified by multiple linear regression analysis.

IV.2. MATERIALS AND METHODS

IV.2.1. Barley malt samples and malt analyses

Nine commercial barley (*Hordeum vulgare* L.) pilsner malt varieties were obtained from two industrial malthouses (malthouse A and B). Astoria, Optic, Pasadena, Prestige and Scarlett are two-row spring barley varieties and Esterel is a six-row winter barley variety. Malt and wort analyses (colour, pH, degree of modification, degree of homogeneity, extract content, extract difference, total and soluble protein content and moisture content) were performed according to the standard methods as defined by the European Brewery Convention (EBC Analytica, 1998). The analytical data of the malt and wort analyses are shown in table IV.1 and are generally within brewery specifications.

IV.2.2. Wort production

Standard laboratory Congress wort was produced according to EBC-Analytica (1998) method 4.5.1. Barley malts were ground in a Bühler-Miag malt mill (Bühler-Miag, Minneapolis, MN) set for fine grist coarseness (0.2 mm gap between the grinding discs). Laboratory-scale mashing experiments were carried out in an automated mashing bath (LB8 Electronic

mashing bath, Funke Gerber GmbH, Berlin, Germany). Ground malt (50.0 g) was mixed with 200 mL water at 46 °C. A temperature of 45 °C was maintained in the mash for 30 min. Then the temperature was raised 1 °C a minute for 25 min. When 70 °C was reached, 100 mL water of 70 °C was added. The temperature was maintained at 70 °C for 1 h before cooling to room temperature in 10 to 15 min. The mash was continuously stirred at 200 rpm. After adjusting the beaker content to 450 g, the mash was filtered over a folded filter (MN 614 ¼ 32 cm diameter, Macherey-Nagel GmbH). To analyse the content of water-extracted HCA's in barley malt samples, the same procedure was applied but before mashing-in the ground barley malt samples were placed in an oven at 130 °C for 5 h according to Debyser *et al*. (1997c) to eliminate enzyme activity.

Table IV.1. *Analytica-EBC malt and wort analyses* [a]

Barley Malt	Md	Hg	E	E_{diff}	M	pH	C	P_t	P_s	KI
Astoria A	88.0	73.5	81.0	2.0	7.85	5.89	5.5	11.5	4.1	35.3
Scarlett A	99.5	97.0	83.3	0.5	5.30	5.89	5.5	10.2	5.0	49.0
Pasadena A	94.6	75.6	82.6	1.8	7.56	5.59	4.1	10.7	4.5	41.7
Optic A	95.4	83.6	82.5	1.1	4.60	6.09	6.1	9.6	3.4	35.0
Prestige A	91.8	70.9	81.3	0.7	5.98	5.96	3.5	10.7	4.3	40.2
Esterel A	93.9	73.6	81.8	2.2	6.37	5.98	3.7	10.2	4.2	41.6
Scarlett B	94.4	78.9	81.8	0.9	5.11	6.03	3.8	10.2	4.2	41.2
Optic B	76.5	64.7	82.5	1.8	5.00	6.13	5.3	9.7	3.6	36.9
Prestige B	93.4	81.5	81.0	0.3	5.12	5.99	3.8	10.6	4.2	39.3

[a] Md = degree of modification (%); Hg = degree of homogeneity (%); E = extract content (% dry malt); E_{diff} = extract difference (% of dry malt); M = moisture content (%); C = colour (EBC units); P_t = total protein content (% of dry malt); P_s = soluble protein content (% of dry malt); KI = Kolbach Index (P_s/P_t)

IV.2.3. Total alkali-extractable HCA's contents in wort and barley malt

Total alkali-extractable HCA's contents in wort and barley malt were determined according to the procedure described in paragraph III.2.2. Instead of 5.0 mL beer, 5.0 mL wort or 100 mg finely ground malt (0.1 mm gap between the grinding discs) were used.

IV.2.4. Quantification of HCA's in wort and barley malt extracts

Quantification of HCA's in wort was performed by HPLC-ECD analysis as described in chapter II.

IV.2.5. Determination of xylose content in wort

Since arabinose present in wort can originate both from AX and arabinogalactan and up to equal amounts of both can be present in wort, the xylose content was used as a relative

measure for AX levels according to Debyser *et al.* (1997c). For the quantification of total xylose content in wort, 2.5 mL 4 M trifluoroacetic acid (TFA) was added to 2.5 mL filtered wort and the mixture was incubated at 100 °C for 1 h. After incubation, the medium was cooled down and TFA was evaporated by flushing the medium with nitrogen at 85 °C. The remaining residue was diluted to 50 mL. Prior to monosaccharide determination by high performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD), samples were deionised by passing them over an anion and cation exchange resin. Monosaccharides were analysed on a Dionex DX500 chromatography system (Dionex Corporation, Sunnyvale, CA) consisting of a Rheodyne model 9125 automatic sample injector (10 µL Sample loop), a Dionex AS40 autosampler, GP40 gradient pump and ED40 electrochemical detector with a gold working electrode. The data collection rate was set at 2 Hz. Separations were performed on a CarboPac PA1 4 x 50 mm guard column and a CarboPac PA1 4 x 250 mm analytical column. Elution was with 16 mM NaOH for 15 min followed by a 10 min column regeneration with 200 mM NaOH. Mobile phases were prepared with highly purified Milli-Q water and low carbonate NaOH (50 %) (J.T. Baker, Phillipsburg, NJ, USA). Mobile phases were degassed with helium prior to use. For detection and quantification, the following pulse potentials (*vs.* Ag/AgCl) and durations were applied to the working electrode: E_1 +0.1 V and 0.40 s, E_2 –2.0 V and 0.02 s, E_3 +0.6 V and 0.01 s and E_4 –0.1 V and 0.06 s. Monosaccharides were identified by coelution with standard reference compounds and quantified by comparison of the peak areas with the peak areas of known concentrations of pure sugars. D(-)-fructose, D(+)-xylose, D(-)-arabinose and D(+)-galactose were obtained from Sigma-Aldrich (Bornem, Belgium). D(+)-glucose was purchased at Merck Eurolab (Darmstadt, Germany). The total xylose content was compensated for free xylose present in wort by omitting the hydrolysis step and analysis of a non-hydrolysed sample.

IV.2.6. Measurement of endoxylanase (EC 3.2.1.8) activity

Samples (3.00 g) of ground malt were suspended in 0.025 M sodium acetate, pH 4.7 (10.0 mL). After 15 min of vigorous shaking, the suspension was centrifuged (3000 g, 15 min). The supernatant was filtered through 0.45 µm PVDF syringe filters (Alltech, Deerfield, IL, USA). Endoxylanase activity assays of malted barley extracts were performed according to Debyser *et al.* (1997a). The extract (1.0 mL) was incubated for 5 min at 50 °C, before adding an azurine-crosslinked (AZCL) wheat AX tablet (Xylazyme AX tablets, Megazyme, Australia). The incubation was then continued for 1 h at 50 °C. The reaction was terminated by adding 1

% w/v Trizma base (tris-(hydroxymethyl)amino-methane) (10.0 mL) and vigorous vortex stirring. After 5 min at room temperature, the tubes were shaken vigorously and the contents filtered through a 0.45 μm PVDF syringe filter (Alltech, Deerfield, IL, USA). The absorbance was measured at 590 nm against a control, which was prepared by incubating the extract without the substrate tablet. A correction was made for the non-enzymic color release by the AZCL-AX tablet. Activities were expressed in ΔA_{590} per gram dry malt per hour.

IV.2.7. Measurement of β-D-xylosidase (EC 3.2.1.37) and α-L-arabinofuranosidase (EC 3.2.1.55) activity

p-Nitrophenyl glycosides were used as substrates to measure β-D-xylosidase and α-L-arabinofuranosidase activities according to the procedure of Cleemput *et al.* (1995b). Samples (3.00 g) of ground malt were suspended in 0.050 M MES (2(N-Morpholino)ethane sulfonic acid), pH 6.0 (10.0 mL). After 15 min of vigorous shaking, the suspension was centrifuged (3000 g, 15 min). The supernatant was filtered through 0.45 μm PVDF syringe filters (Alltech, Deerfield, IL, USA). The activity was measured as follows: *p*-nitrophenyl-α-L-arabinofuranoside and *p*-nitrophenyl-β-D-xylopyranosidase (Sigma, St. Louis, MO) solutions (10 mM) were prepared in a 50 mM MES buffer, pH 6.0. Aliquots of these solutions (100 μL) were incubated with 50 μL of barley malt extract. The reaction was stopped after 30 min at 40 °C by adding 1.5 mL a 1 % w/v Trizma base solution. In a control assay, Trizma base was added before the malt extract. The release of *p*-nitrophenol from the *p*-nitrophenyl glycoside was determined colorimetrically at 410 nm. Activities were expressed as units per gram dry malt. One enzyme unit (EU) was defined as the amount of enzyme that released 1 μmol *p*-nitrophenol from the substrate per minute at 40 °C and pH 6.0.

IV.2.8. Measurement of cinnamoyl esterase (EC 3.1.1.73) activity

Cinnamoyl esterase activity in barley malt extracts was assessed spectrophotometrically using 4-nitrophenyl ferulate (4NPF) as a substrate based on a method adapted from Mastihuba *et al.* (2002). 4NPF was obtained from the Faculty of Chemical Technology, Slovak Universtity of Technology, Bratislava, Slovakia. Samples (3.00 g) of ground malt were suspended in 0.050 M MES, pH 6.0 (10.0 mL). After 15 min of vigorous shaking, the suspension was centrifuged (3000 g, 15 min). The supernatant was filtered through 0.45 μm PVDF syringe filters (Alltech, Deerfield, IL, USA). The substrate solution was prepared by mixing 9 volumes of 0.1 M potassium phosphate buffer solution, pH 6.0, containing 2.5 % Triton X-100 with 1

volume of 10.0 mM 4NPF in DMSO followed by immediate vortexing. The buffer-4NPF solution was prepared freshly before analysis. The DMSO solution of 4NPF was prepared within 24 h and kept at room temperature. The reaction mixture comprised 0.4 mL substrate solution and 1.2 mL barley malt extract. A blank sample was included using water instead of barley malt extract to correct for the non-enzymic color release of the substrate. Samples were incubated at 30 °C for 2 h. The release of *p*-nitrophenol from 4NPF was determined colorimetrically at 410 nm. The difference in absorbance (against the control sample) before and after incubation was used to calculate enzyme activity. Activities were expressed as EU per gram dry malt. One EU was defined as the amount of enzyme that released 1 nmol *p*-nitrophenol from the substrate per minute at 30 °C and pH 6.0.

Cinnamoyl esterase activity *versus* the methyl esters of pCA (MpCA), FA (MFA) and SA (MSA) were determined by incubating 3 mL malt extract (prepared as described above) with 1 mL of substrate solution for 2 h at 30 °C. The substrate solution was prepared by dissolving the HCA methyl esters in methanol (80 mM) and diluting these solutions to 4 mM with 50 mM MES pH 6.0. After incubation, the reaction was stopped by adding 0.8 mL glacial acetic acid and samples were frozen (-18 °C) until HPLC-ECD analysis. A blank sample was obtained by adding the substrate solution after the addition of acetic acid. The HCA methyl esters were tested both separately and in a mixture containing all three of them together (26.7 mM of each in the stock solution). Activities were expressed as EU per gram malt. One EU was defined as the amount of enzyme that released 1 nmol HCA's from the substrate under the conditions specified.

IV.2.9. Isolation of WEAX from barley malt and endoxylanase treatment

WEAX was isolated from barley malt (Scarlett A) according to a procedure adapted from Cleemput *et al*. (1995b). The isolation was carried out at room temperature unless indicated otherwise. Barley malt (1.0 kg) was ground in a Bühler-Miag malt mill (Bühler-Miag, Minneapolis, MN) set for fine grist coarseness (0.2 mm gap between the grinding discs). The ground malt was placed in an oven at 130 °C for 5 h to eliminate enzyme activities, extracted with water (5:1 v/w; 15 min; 30 °C) and filtered over a folded filter (MN 614 ¼ 32 cm diameter). The supernatant was heated to 90 °C and residual starch was hydrolysed by addition of α-amylase solution (2.0 mL, Type XII-A from *Bacillus licheniformis*, A3403, Sigma Chemical Co, St. Louis, MO). The mixture was incubated at 90 °C for 120 min, cooled to room temperature and filtered again over a folded filter (MN 614 ¼ 32 cm

diameter). The WEAX was precipitated by stepwise addition of ethanol (96 %) to a final concentration of 65 % v/v. The mixture was stirred for 30 min, kept at 4 °C overnight and recovered by centrifugation (10,000 g; 30 min; 4 °C). The precipitate was dissolved in water (1.0 L) and ethanol was added to a final concentration of 65 % v/v. The mixture was stirred for 30 min; kept at 4 °C overnight and recovered by centrifugation (10,000 g; 30 min; 4 °C). The precipitate was washed with ethanol (500 mL) and with acetone (500 mL) with intermediate stirring (120 min) and centrifugation (10,000 g; 30 min; 4 °C). The final pellet was dried for 24 h at 45 °C. With this procedure, 58 % of total WEAX present in the Scarlett barley malt (0.45 % w/w) was recovered. The resulting precipitate contained 7.5 % protein and had a final AX content of 94 % on carbohydrate basis. Contaminating sugars consisted predominantly of galactose and glucose, originating from arabinogalactan and glucan. The isolated WEAX contained 0.12 % w/w esterbound FA.

An amount of WEAX preparation, corresponding to 30 % of esterbound FA initially present in Scarlett A wort, was dissolved in 0.05 M NaAc pH 4.7 (20.0 mL) and incubated with endoxylanase (50.0 mg) from *T. viride* (Fluka, St. Louis, MO) for 60 min at 45 °C. Enzyme activity was eliminated by heating the sample for 15 min at 100 °C. The efficiency of the endoxylanase treatment in reducing the molecular weight of the WEAX fragments could be evaluated by a clear visible reduction in viscosity of the solution after the procedure. A blank sample was made by adding the endoxylanase during the boiling step. HPLC-ECD analysis showed that during the treatments no release of esterbound FA did occur in any of the two samples. Both aliquots were added at the start of Congress wort production with Scarlett A barley malt according to the procedure specified above.

IV.2.10. Statistical Analysis

All analyses were carried out at least in duplicate (true replicates). Results are represented as mean ± standard deviation and considered significantly different at a 95 % confidence level (*t*-test statistic; $p < 0.05$). The data obtained from the barley malt and wort analyses were analysed using Multiple Linear Regression (MLR). The general purpose of MLR is to study the relation between a dependent (response) variable (Y) and other independent (explanatory) variables (X_n). The general mathematical function is a first-degree equation specified as:

$$Y = \alpha + \beta_1 X_1 + \beta_2 X_2 + \ldots + \beta_m X_m + \varepsilon$$

where X_m represents the m experimental variables tested, α is the constant term, and ε the predictive error. β_m represents the regression coefficients of the independent variables, and each represents the "weight" (correlation) of the respective independent variable. To measure the strength of relationship between the measured variables, Pearson's correlation coefficients (r) were calculated. The statistical package, XLSTAT 2006 Version 2006.06 (Addinsoft, Paris, France) was used for the statistical calculations.

IV.3. RESULTS AND DISCUSSION

IV.3.1. Release of free and esterbound HCA's from malted barley during wort production

The amount of total alkali-extractable pCA and FA in 9 malted barley varieties from two malthouses and their corresponding worts are represented in table IV.2. The values represent the sum of the HCA's either present in free or in esterbound form in barley malt and in wort. Mean values with no common letters differ significantly at the 95 % confidence level.

Table IV.2. *Total alkali-extractable pCA and FA in 9 malted barley varieties and their corresponding worts. Mean values with no common letters differ significantly at the 95 % confidence interval*

Barley malt	MALT		WORT		% in WORT	
	pCA (µg/g dm)	FA (µg/g dm)	pCA (µg/g dm)	FA (µg/g dm)	pCA	FA
Astoria A	267 ± 4 ^{d}	580 ± 28 ^{b,c}	10.4 ± 0.6 ^{d,e}	62.9 ± 3.9 ^{c,d}	4.0	10.8
Scarlett A	252 ± 4 ^{c,d}	532 ± 37 ^{a,b}	13.0 ± 0.7 ^{f}	63.7 ± 2.0 ^{d}	5.1	12.0
Pasadena A	263 ± 10 ^{d}	597 ± 18 ^{c}	6.1 ± 0.3 ^{a}	42.4 ± 1.3 ^{a}	2.3	7.1
Optic A	360 ± 12 ^{f}	634 ± 30 ^{c,d}	8.4 ± 0.6 ^{b,c}	54.7 ± 0.7 ^{b}	2.3	8.6
Prestige A	294 ± 11 ^{e}	649 ± 14 ^{d}	9.0 ± 0.4 ^{c,d}	59.0 ± 1.1 ^{c}	3.1	9.1
Esterel A	215 ± 8 ^{a,b}	653 ± 12 ^{d}	7.2 ± 0.8 ^{a,b}	64.6 ± 0.3 ^{d}	3.3	9.9
Scarlett B	236 ± 10 ^{b,c}	563 ± 8 ^{b}	12.5 ± 0.8 ^{f}	70.1 ± 1.6 ^{e}	5.3	12.5
Optic B	239 ± 10 ^{b,c}	481 ± 16 ^{a}	10.4 ± 0.6 ^{d,e}	57.2 ± 2.3 ^{b,c}	4.3	11.9
Prestige B	204 ± 11 ^{a}	646 ± 13 ^{d}	10.6 ± 0.6 ^{e}	55.6 ± 1.9 ^{b,c}	5.2	8.6

Total alkali-extractable FA and pCA content in malt ranged from 481-653 µg/g dry malt and 204-360 µg/g dry malt respectively, making FA the predominant phenolic acid in barley malt. These amounts are comparable with those found in unmalted barley by Hernanz *et al.* (2001), which were found to be 359-624 µg/g and 79-260 µg/g for total alkali-extractable FA and pCA, respectively. The degree in variability for the pCA and FA content in malt was 18 %

and 10 %, respectively. Significant differences were observed between the total alkali-extractable HCA's contents of the different barley varieties. Differences were also observed between subsequent harvesting years (results not shown). Possibly, smaller grains contain higher levels of HCA's because of the higher surface-to-volume ratio leading to a higher percentage of outer layers. However, no correlation was found between the 1000-grain-weight of different barley cultivars and their FA concentration (Zupfer *et al.*, 1998). The HCA's contents in barley malt may not only depend on genotypic variations, but also on environmental factors (growth environment, temperature stress, water excess, drought, solar radiation,...) and post harvest history. Both genotype, growing environment and their interaction have been known to influence phenolic profiles of wheat and rye (Moore *et al.*, 2006). Zupfer *et al.* (1998) found phenolic acid concentrations to correlate well with agronomic factors. Variations in the steeping, germination and kilning regime can partly explain the difference in HCA's contents for identical malt varieties originating from the two different malthouses. Steeping and germination time and conditions have been known to influence total FA levels in wheat grains (Yang *et al.*, 2001). Fry *et al.* (1979) showed that the use of gibberellic acid to induce germination stimulates the synthesis of FA. They observed both an increase in soluble and macromolecule esterified FA levels in cell cultures of *Spinacia oleracea* L., Maillard and Berset (1995) showed that the amount of insoluble bound HCA's in barley malt are influenced by the kilning regime.

The release of 6.1-13.0 µg/g dry malt pCA and 42.4-70.1 µg/g dry malt FA from malted barley during Congress wort brewing corresponded to 0.70-1.53 ppm pCA and 4.90-8.32 ppm FA present in wort (either in free or in esterbound form). The degree in variability in total alkali-extractable pCA and FA content in wort (22 % and 13 %, respectively) were higher than those in malt. During mashing, only 7.1-12.5 % of total alkali-extractable FA in barley malt was released into wort (either in free or esterbound form), leaving the majority of esterified FA in the spent grain. Concerning the total alkali-extractable pCA in malt, only 2.3-5.3 % was transferred to wort. No correlation was found between total alkali-extractable malt and total wort HCA's content. This is probably due to variations in WEAX-content and differences in AX-degrading enzyme activities between different barley malt cultivars. Woffenden *et al.* (2002) showed that, regardless of the kilning regime, also the initial moisture content of the green malt has an important effect on the level of FA in water-extracts of barley malt.

IV.3.2. Free *versus* esterbound hydroxycinnamic acids in wort

The combined activity of AX-hydrolysing enzymes leads to the release of both free and AX-esterified HCA's in wort. Only free HCA's are available for decarboxylation by the yeast later in the brewing process. HPLC-ECD analysis of wort before and after alkaline hydrolysis permits to determine the amount of pCA and FA either present in free form or AX-esterbound form (table IV.3).

Table IV.3. *Release of free and esterbound pCA and FA in Congress wort from 9 malted barley varieties (ppm) Mean values with no common letters differ significantly at the 95 % confidence interval*

	FREE		ESTERBOUND		% FREE	
Barley Malt	**pCA (ppm)**	**FA (ppm)**	**pCA (ppm)**	**FA (ppm)**	**pCA**	**FA**
Astoria A	0.88 ± 0.03 ^{d}	3.12 ± 0.04 ^{e}	0.31 ± 0.08 ^{a,b}	4.13 ± 0.45 ^{a,b}	74.0	43.1
Scarlett A	1.10 ± 0.03 ^{e}	2.23 ± 0.09 ^{c}	0.44 ± 0.09 ^{b}	5.31 ± 0.26 ^{c,d}	71.5	29.5
Pasadena A	0.53 ± 0.04 ^{a}	0.95 ± 0.19 ^{a}	0.17 ± 0.05 ^{a}	3.95 ± 0.25 ^{a}	75.7	19.3
Optic A	0.54 ± 0.05 ^{a}	1.81 ± 0.06 ^{b}	0.46 ± 0.09 ^{b}	4.71 ± 0.11 ^{b}	53.7	27.8
Prestige A	0.76 ± 0.02 ^{c}	2.46 ± 0.13 ^{d}	0.30 ± 0.06 ^{a,b}	4.48 ± 0.18 ^{b}	71.2	35.5
Esterel A	0.67 ± 0.01 ^{b}	1.83 ± 0.17 ^{b}	0.17 ± 0.09 ^{a}	5.73 ± 0.17 ^{d}	79.3	24.3
Scarlett B	1.20 ± 0.05 ^{f}	3.45 ± 0.26 ^{e}	0.29 ± 0.10 ^{a,b}	4.87 ± 0.32 ^{b,c}	80.8	41.5
Optic B	0.73 ± 0.03 ^{c}	2.35 ± 0.08 ^{c,d}	0.50 ± 0.12 ^{b}	4.43 ± 0.28 ^{a,b}	59.7	34.7
Prestige B	0.78 ± 0.07 ^{c,d}	2.05 ± 0.19 ^{b,c}	0.48 ± 0.10 ^{b}	4.55 ± 0.30 ^{a,b}	62.2	31.0

Significant differences in both free and esterbound pCA and FA levels between worts from 9 barley malt varieties were observed. Especially concerning free pCA and FA levels, a high degree of variability was observed (25.7 % and 31.0 %, respectively). Levels of free phenolic acids in wort varied from 0.95 to 3.45 ppm for FA and from 0.53 to 1.20 ppm for pCA. The majority of pCA occured in free form (53.7-80.8 % of total wort pCA) whereas FA is mainly esterbound to AX as only 19.3-43.1 % of total wort FA occured in free form. However, because total wort FA levels were higher than total pCA concentrations, FA is the most abundant free phenolic acid in wort.

IV.3.3. Water-extracted *versus* enzymatically released free HCA's in wort

The amount of free pCA and FA was determined both in normal Congress wort and in wort produced according to the Congress wort procedure but using malts without enzyme activity. HPLC-ECD analysis of both worts allows to discriminate between the water-extracted and the enzymatically solubilised free HCA's. The results are represented in table IV.4.

Table IV.4. *Water-extracted* versus *enzymatically released free pCA and FA in Congress wort from 9 malted barley varieties. Mean values with no common letters differ significantly at the 95 % confidence interval*

	WATER-EXTRACTED		ENZYME RELEASED		% ENZYME RELEASED	
Barley malt	**pCA (ppm)**	**FA (ppm)**	**pCA (ppm)**	**FA (ppm)**	**pCA**	**FA**
Astoria A	0.33 ± 0.05 ^{a,b,c}	0.73 ± 0.04 ^{b}	0.55 ± 0.06 ^{c}	2.39 ± 0.06 ^{g}	62.6	76.5
Scarlett A	0.38 ± 0.05 ^{b,c}	0.67 ± 0.09 ^{a,b}	0.72 ± 0.05 ^{d}	1.55 ± 0.05 ^{e}	65.3	69.8
Pasadena A	0.24 ± 0.08 ^{a,b}	0.51 ± 0.04 ^{a}	0.29 ± 0.09 ^{a,b}	0.43 ± 0.09 ^{a}	54.7	45.7
Optic A	0.29 ± 0.01 ^{a}	0.69 ± 0.01 ^{b}	0.25 ± 0.05 ^{a}	1.12 ± 0.05 ^{c}	47.0	61.9
Prestige A	0.27 ± 0.08 ^{a,b,c}	0.64 ± 0.07 ^{a,b}	0.49 ± 0.08 ^{b,c}	1.82 ± 0.08 ^{f}	64.6	73.9
Esterel A	0.47 ± 0.01 ^{d}	0.94 ± 0.03 ^{c}	0.19 ± 0.01 ^{a}	0.90 ± 0.01 ^{b}	28.6	48.9
Scarlett B	0.39 ± 0.02 ^{c}	0.53 ± 0.02 ^{a}	0.81 ± 0.05 ^{d}	2.92 ± 0.05 ^{h}	67.6	84.6
Optic B	0.30 ± 0.04 ^{a,b,c}	0.75 ± 0.09 ^{b}	0.43 ± 0.04 ^{b}	1.60 ± 0.04 ^{e}	58.7	68.1
Prestige B	0.29 ± 0.02 ^{a}	0.78 ± 0.11 ^{b}	0.49 ± 0.07 ^{b,c}	1.26 ± 0.07 ^{d}	62.9	61.8

Of total free wort pCA, 28.6 % to 67.6 % is enzymatically released compared to 48.9 % to 84.6 % for FA. This corresponds to wort levels of 0.19-0.81 ppm pCA and 0.43-2.92 ppm FA, which are enzymatically released from AX-esterified forms by cinnamoyl esterase activity during mashing. Water-extracted free FA levels in wort range between 0.53 and 0.94 ppm corresponding with 4.08-7.52 µg/g free FA in barley malt. These values are higher than the free FA levels found in water-extracts of unmalted barley (2.51-3.87 µg/g) by Zhao *et al.* (2006). Samaras *et al.* (2005) also found higher free FA concentrations in pale malt and lager malt than in unmalted barley. Probably this is due to cinnamoyl esterase activity during the germination and the first stages of the green malt kilning.

In summary, the distribution of FA between malt, wort and spent grain and between free and bound forms is represented in figure IV.1. Because of their bifunctional nature, phenolic acids may form both ester and ether linkages with various biopolymers through interaction of their carboxyl and phenolic groups (figure I.8). However, since no enzymes are known to act on this etherbound fraction and chemical hydrolysis of these linkages requires harsh conditions, etherbound FA present in malt will be integrally transferred to the spent grains. On the contrary, FA present in free form in malt will be water-extracted during mashing and passed on into the wort. Concerning the fraction of FA, which is esterbound to AX, almost 90 % will end up into the spent grain. On average, 10 % of esterbound FA in malt will be transferred to wort: 6-9 % remains esterbound to water-extracted or solubilised AX and 1-4 % is being released by malt cinnamoyl esterase activity into its free form. The combined fraction of both the water-extracted and the enzymatically released free FA can be converted to 4VG during wort boiling or fermentation.

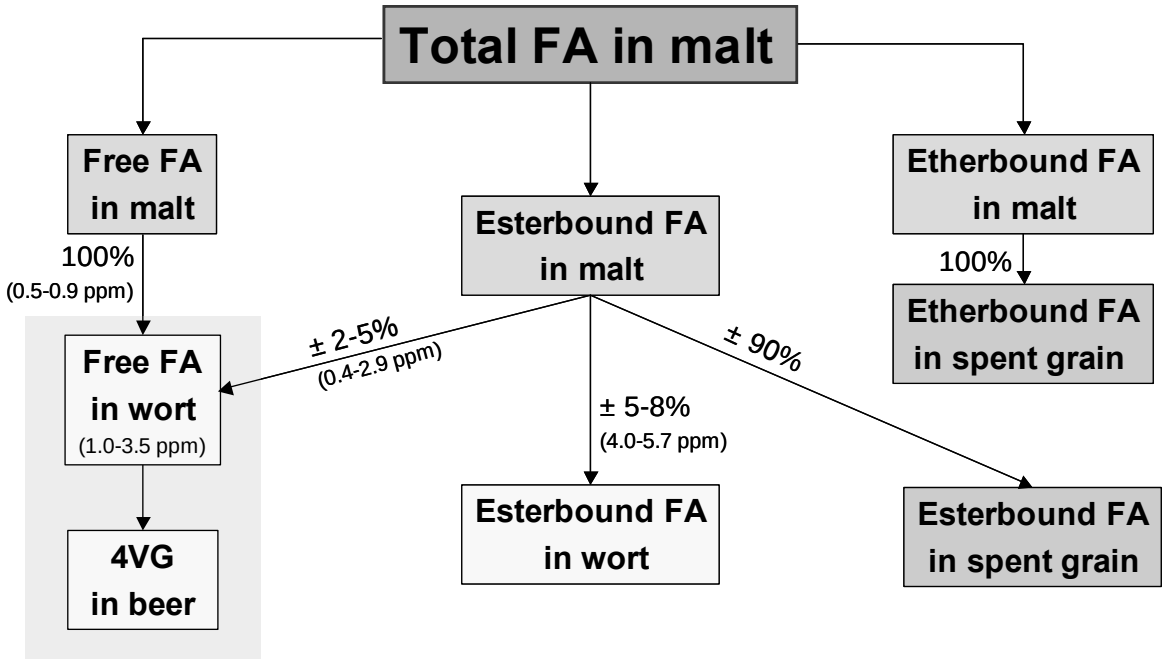


Figure IV.1. *Fractionation of FA during wort production: distribution of free, esterbound and etherbound FA in wort and spent grain*

IV.3.4. Esterbound HCA's and AX in wort

In table IV.5, wort polymeric xylose levels are presented together with the total amount of pCA and FA initially released in wort in esterbound form. The latter can be calculated as the difference between the total wort HCA's contents and the water-extracted HCA's contents, or as the sum of the enzymatically released HCA's (present in free form in wort) and the final esterbound HCA's levels present in wort after mashing.

Table IV.5. *Polymeric xylose content (g/L) and initially esterbound pCA and FA content (ppm) in Congress wort made with 9 malted barley varieties*

Barley Malt	Polymeric Xylose (g/L)	INITIALLY ESTERBOUND pCA (ppm)	FA (ppm)
Astoria A	0.629 ± 0.032	0.86 ± 0.09	6.51 ± 0.45
Scarlett A	0.766 ± 0.031	1.15 ± 0.10	6.87 ± 0.26
Pasadena A	0.452 ± 0.063	0.46 ± 0.09	4.39 ± 0.15
Optic A	0.575 ± 0.006	0.72 ± 0.07	5.83 ± 0.09
Prestige A	0.552 ± 0.008	0.79 ± 0.10	6.29 ± 0.15
Esterel A	0.585 ± 0.014	0.36 ± 0.09	6.62 ± 0.05
Scarlett B	0.846 ± 0.015	1.11 ± 0.09	7.79 ± 0.19
Optic B	0.605 ± 0.064	0.89 ± 0.12	6.04 ± 0.28
Prestige B	0.626 ± 0.076	0.97 ± 0.08	5.81 ± 0.26

Good correlations can be found between wort xylose levels and these esterbound pCA and FA levels. Pearson's correlation coefficients (*r*) were 0.76, 0.95 and 0.98 for pCA, FA and their sum, respectively. Hence, a clear correlation between (either water-extracted or solubilised) wort AX and HCA's being released in esterbound form into the wort can be found. From the amount of xylose present in the wort and either the total amount of pCA and FA being initially released into the wort in esterbound form or the amount of esterbound pCA and FA present in wort at the end of the mashing process, the Xyl:pCA and Xyl:FA ratios of AX before and after cinnamoyl esterase action can be calculated, respectively. The results are presented in table IV.6. Esterel, the only six-row barley variety included in this study, had outlier values for pCA and was excluded from the calculations. Initially, one pCA and one FA molecule was bound every 796 and 128 xylose residues, respectively. Hence, the larger amount of FA being transferred to wort in esterbound form compared to pCA is due to FA being bound in higher quantities to AX than pCA. The variabilities between the Xyl:pCA and Xyl:FA ratios of AX released from different malt varieties (10 % and 9 %, respectively) are quite low, indicating that, initially, AX released from different barley malt varieties were quite similar in structure.

Table IV.6. *Xyl:pCA and Xyl:FA ratios (mol:mol) of wort AX before and after cinnamoyl esterase activity in Congress wort from 9 malted barley varieties. The ratio is a measure of the average number of xylose molecules per pCA or FA molecule in the AX backbone*

	START		END	
Barley Malt	**Xyl:pCA**	**Xyl:FA**	**Xyl:pCA**	**Xyl:FA**
Astoria A	797	125	2211	197
Scarlett A	679	135	1792	174
Pasadena A	834	104	2256	115
Optic A	878	128	1356	158
Prestige A	900	134	2341	189
Esterel A	(1760) [a]	114	(3689) [a]	132
Scarlett B	834	141	3241	225
Optic B	742	130	1334	177
Prestige B	707	139	1438	178
mean ± stdev	796 ± 80	128 ± 12	1996 ± 653	172 ± 33
Variability (%)	10	9	33	19

[a] *outlier: result omitted from calculation*

At the end of the mashing process, the Xyl:pCA and the Xyl:FA ratios were significantly higher (1996 and 172, respectively) due to the action of the cinnamoyl esterase enzyme. This corresponds with, on average, 60 % of initially esterbound pCA and 23.5 % of initially

esterbound FA in wort being hydrolysed. The increased values of the variabilities of both ratios (33 % and 19 %, respectively) reflect distinct variations in cinnamoyl esterase enzyme activities between the different malt varieties.

IV.3.5. Release of HCA's from their methyl esters by barley malt extracts

Cinnamoyl esterase activity of three barley malt (Astoria A, Scarlett A and Optic B) extracts was assessed both on MpCA, MFA and MSA separately and on the three methyl esters together. Results are represented in table IV.7.

Table IV.7. *Release of HCA's (EU/g malt) by barley malt extracts: (A) MpCA, MFA and MSA administered separately (80 mM in stock solution) and (B) MpCA, MFA and MSA administered together (3 x 26.7 mM in stock solution)*

Barley Malt	pCA	FA	SA
(A) Astoria A	1.41 ± 0.11	1.76 ± 0.06	0.74 ± 0.03
Scarlett A	0.65 ± 0.02	1.04 ± 0.05	0.51 ± 0.02
Optic B	1.55 ± 0.03	2.18 ± 0.05	0.94 ± 0.02
(B) Astoria A	0.52 ± 0.03	0.36 ± 0.04	0.14 ± 0.01
Scarlett A	0.37 ± 0.01	0.26 ± 0.01	0.16 ± 0.01
Optic B	0.98 ± 0.06	0.58 ± 0.03	0.30 ± 0.01

When the enzyme activity was measured against each of the three methyl esters separately, the activity was the highest on MFA, followed by MpCA and then MSA for all three malt varieties corresponding with the results obtained by Bartolomé *et al.* (1996). However, when the three methyl esters were administered in the same assay, more pCA was being released followed by FA and SA. Apparently, once bound to the active site of the cinnamoyl esterase enzyme, the turnover of esterbound FA was faster than that of esterbound pCA and SA. However, it seems that pCA was the substrate being preferentially bound by the active site of the enzyme compared to FA when both substrates are present together. This can explain why pCA is the HCA being preferentially released from its bound forms during mashing (60 % compared to 23.5 % for FA).

IV.3.6. Activity of AX-degrading enzymes in barley malt extracts

The activity of four AX degrading enzymes (endoxylanase, β-D-xylosidase, α-L-arabinofuranosidase and cinnamoyl esterase) in barley malt extracts are represented in table IV.8.

Table IV.8. *Endoxylanase, xylosidase, arabinofuranosidase and cinnamoyl esterase activities in barley malt extracts from 9 malted barley varieties*

Barley malt	Endoxylanase (ΔA/g malt/h)	Xylosidase (EU/g malt)	Arabinofuranosidase (EU/g malt)	Cinn. esterase (EU/g malt)
Astoria A	0.430 ± 0.014	0.213 ± 0.008	0.087 ± 0.003	2.72 ± 0.10
Scarlett A	0.310 ± 0.021	0.230 ± 0.004	0.047 ± 0.009	2.17 ± 0.15
Pasadena A	0.313 ± 0.007	0.162 ± 0.012	0.046 ± 0.004	1.99 ± 0.28
Optic A	0.320 ± 0.023	0.211 ± 0.012	0.086 ± 0.001	2.57 ± 0.21
Prestige A	0.394 ± 0.013	0.192 ± 0.002	0.082 ± 0.004	2.61 ± 0.09
Esterel A	0.283 ± 0.012	0.217 ± 0.004	0.078 ± 0.005	1.74 ± 0.14
Scarlett B	0.468 ± 0.006	0.265 ± 0.012	0.096 ± 0.005	3.34 ± 0.17
Optic B	0.292 ± 0.013	0.232 ± 0.008	0.067 ± 0.002	3.12 ± 0.16
Prestige B	0.388 ± 0.006	0.247 ± 0.007	0.073 ± 0.017	2.46 ± 0.10

Apart from the clear differences in enzyme activity between the 9 malted barley varieties, no correlation could be found between any pair of the four enzyme activities ($r_{xy} < 0.63$ for each combination). This is probably due to the fact that the malting process (steeping, germination and kilning) has a different impact on the level of each enzyme. While malt has a considerably higher endoxylanase activity than the corresponding barley cultivar, it is as active in arabinofuranosidase and less active in xylosidase activity (Preece *et al.*, 1958). Barley cinnamoyl esterase activity is known to decrease during germination (Sancho *et al.*, 1999) and since it is relatively heat labile (Humberstone *et al.*, 2000; Sun *et al.*, 2005), it will probably decrease during kilning too.

IV.3.7. Factors influencing the release of FA in wort: MLR analysis

Pearson's correlation coefficients were used to evaluate the relative significance of the malt parameters on the release of free FA in wort. Both enzymatically released FA and water-extracted FA were taken in consideration (table IV.4). As explanatory variables, the activities of the four AX degrading enzymes (table IV.8), degrees of modification and extract content (table IV.1), total malt FA (table IV.2), and total esterbound FA content being released into wort (table IV.5) were included in this study. Results are shown in table IV.9. There were no significant correlations between the amount of water-extracted FA and any of the malt parameters under investigation ($p > 0.05$). Probably, the water-extracted FA levels depend on other characteristics such as environmental conditions, barley growing regime, harvesting conditions and specific processes during barley germination and kilning. On the contrary, better relationships were obtained when the enzymatically released FA was considered. The endoxylanase activity, esterase activity and total esterbound FA levels significantly correlated

to the amount of enzymatically released FA in wort at a 95 % confidence level. No significant correlations between the release of FA and the extract content, the degree of modification and the xylosidase and arabinofuranosidase activities of the different barley malt varieties were observed.

Table IV.9. *Pearson's correlation coefficients (r) between the amount of enzymatically released and the amount of water-extracted FA in Congress wort from 9 malted barley varieties and several malt parameters.*

Malt parameters	Water Extracted FA	Enzyme Released FA
Endoxylanase	-0.392	0.808*
Cinnamoyl esterase	-0.356	0.787*
α-L-Arabinofuranosidase	0.153	0.625
β-D-Xylosidase	0.208	0.615
Degree of modification	-0.219	-0.174
Degree of extract	-0.253	-0.345
Total malt FA	0.244	-0.328
Total esterbound FA	0.126	0.798*

Values with an asterisk (*) are significantly different from 0 with a significance level of α = 0.05

A stepwise MLR analysis was applied to develop a model explaining which factors influence the amount of enzymatically released FA. Corresponding with the previous calculations, three parameters have a regression coefficient in the model that is significantly different from zero (endoxylanase activity $p = 0.036$; esterase activity $p = 0.038$; total esterbound FA $p = 0.010$). The following equation was obtained:

[enzymatically released FA] =
-3.65 + 4.22 [endoxylanase] + 0.53 [cinnamoyl esterase] + 0.38 [total esterbound FA]

The MLR is statistically significant with a degree of significance $p = 0.001$ explaining 92 % of the variation of the amount of FA released (adjusted R^2 taking into account the number of independent variables included in the model) and has a standard error of estimate (root mean squared error) of 0.21. The correlation between the observed and the calculated values of the amount of enzymatically released FA are depicted in figure IV.2. Three new, randomly selected commercial malts were used to validate the model. Endoxylanase, esterase and total esterbound FA levels were determined and the theoretically calculated FA content was compared with the measured value. All three had observed values within the 95 % confidence interval of the model (figure IV.2).

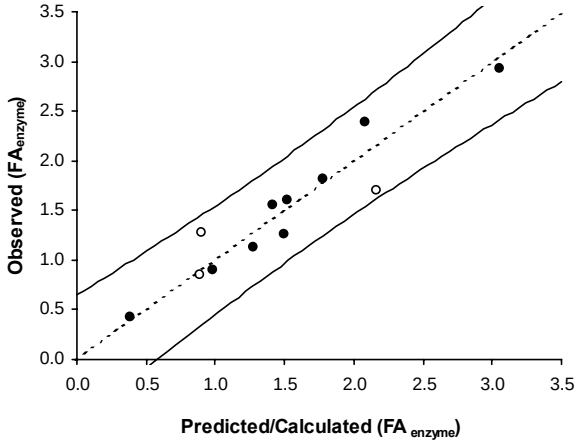


Figure IV.2. *Comparison between observed and calculated enzymatically released FA levels in wort from 9 malted barley varieties (●) and between observed and predicted enzymatically released FA levels in wort from 3 malt test samples (○) (ppm)*

The model suggests that it is not only the activity of the cinnamoyl esterase itself that affects the amount of FA being released into the wort from its esterbound forms. Also the amount of esterbound FA present in the wort and the endoxylanase activity turn out to be important factors. The former represents the substrate of the cinnamoyl esterase enzyme. The latter can affect both the amount of esterase substrate (endoxylanase solubilising activity) as well as the molecular weight of the AX polymers (endoxylanase depolymerising activity). Both effects were mimicked by addition of WEAX (untreated and pretreated with *T. viride* endoxylanase) during wort production. Endoxylanase activity enhancing FA release was already shown by the addition of exogenous microbial AX-degrading enzymes to barley spent grain (Bartolomé *et al.*, 1996) and wort (Szwajgier *et al.*, 2005b). It has already been shown that native barley endoxylanase activity enhances the release of FA from brewers spent grain with extracts from barley and germinated barley (Sancho *et al.*, 2001).

IV.3.8. Addition of isolated barley malt WEAX during wort production

To increase the amount of esterbound FA during wort production, isolated WEAX (corresponding with an increase of 30 % of esterbound FA initially present in wort) were added at the start of the mashing-in process. Increasing the amount of esterbound FA lead to an additional release of FA of 10.2 % (figure IV.3). This demonstrates the dose-response relationship between the amount of FA released by cinnamoyl esterase activity and the amount of esterbound FA present in wort i.e. the substrate of the cinnamoyl esterase enzyme. To evaluate the effect of endoxylanase activity on the enzymatic release of FA during

mashing, an aliquot of the WEAX preparation pretreated with endoxylanase was added during mashing. When the WEAX added to the wort were pretreated with endoxylanase from *T. viride* to lower the molecular weight of the AX polymers, an increase in FA release of 17.7 % versus the blank sample was seen (figure IV.3). Reducing the molecular weight of the AX polymers generates more suitable esterase substrates by reducing the steric hindrance and enhancing the access of the cinnamoyl esterase to esterbound HCA's. The experiment shows a clear correlation between the amount and the molecular weight of the esterase substrate and amount of FA released during brewing.

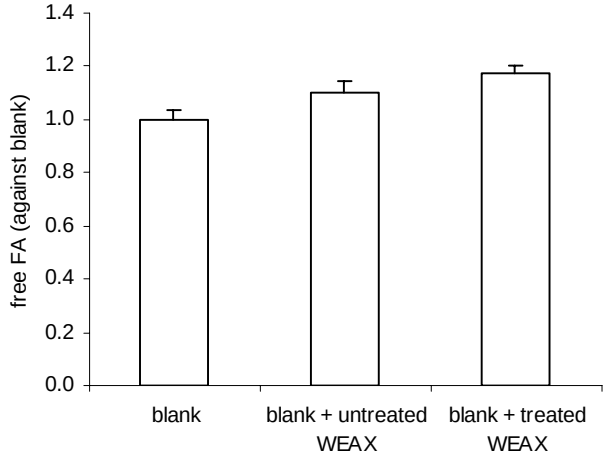


Figure IV.3. *Release of FA (relative concentration versus blank) during wort production: influence of the addition of isolated barley malt WEAX and addition of pretreated barley malt WEAX with endoxylanase from T. viride*

IV.4. CONCLUSION

A large variability in HCA's content between different barley malt varieties and their corresponding worts was observed. Differences were also found between free FA levels from identical malt varieties originating from different malthouses. This demonstrates the importance of selecting a suitable malt variety as the first means of controlling the final volatile phenol levels in beer. It was shown that only a small part of the HCA's in malt is transferred to wort during mashing, the lion's share remaining in the spent grains. Free HCA's in wort are both water-extracted and enzymatically released by cinnamoyl esterase activity. This esterase activity is reflected in the increase of the Xyl:FA and Xyl:pCA ratios during mashing. Esterase activities clearly differ between different barley malt varieties, as

do other AX-degrading enzyme activities. The substrate specificity of the esterase *versus* HCA methylesters reflects the release of HCA's during mashing. MLR analysis suggested that the enzymatic release of FA during mashing does not only depend on the esterase activity but also on the amount of AX-esterbound FA initially present in the wort and on the endoxylanase activity of the barley malt. This was confirmed by the addition of isolated WEAX pretreated with *T. viride* endoxylanase.

CHAPTER V

INFLUENCE OF PROCESS PARAMETERS AND GRIST COMPOSITION ON FERULIC ACID RELEASE AND 4-VINYLGUAIACOL FORMATION DURING BREWING

In this chapter, the effect of mashing variables such as mashing-in temperature, time and pH, mash thickness, grist coarseness and composition, and stirring regime on the release of ferulic acid were examined. Given one barley malt variety, the multitude of choice in setting various process parameters during brewhouse operations can give rise to worts with widely varying ferulic acid levels. The conversion of ferulic acid to its corresponding volatile phenol 4-vinylguaiacol during brewhouse operations was found to be of minor importance.

V.1. INTRODUCTION

In the previous chapter, significant differences in free and esterbound HCA's release between different malted barley (*Hordeum vulgare* L.) varieties were observed indicating that the choice of a suitable barley malt variety is the first step in controlling HCA's release during brewing. However, given one barley malt variety, the multitude of choice in the setting of various process parameters during brewhouse operations may give rise to worts with varying concentrations of POF precursors and, hence, phenolic flavour potential in the final beer. The objectives of this chapter were to examine the release of free and esterbound FA and the thermal decarboxylation of FA to 4VG during brewhouse operations. The influence of various process parameters (mashing-in temperature, time and pH; mash thickness; grist coarseness and composition; stirring regime) were studied. The T,t-dependence of the activity of AX degrading enzymes (and more specifically the activity of the cinnamoyl esterase) were correlated with free and esterbound ferulic acid release during mashing. Finally, a pilot-scale (5 hL) wort production process was conducted to validate laboratory-scale mashing experiments.

V.2. MATERIALS AND METHODS

V.2.1. Materials

In the laboratory-scale mashing experiments, a two-row pilsner barley malt variety Scarlett (5 EBC) from Cargill Malt Division (Herent, Belgium) was used. Legat, Limes, Patrel, Meunier, Drifter, Alsace, Winnetou, Biscay, Pulsar and Nijinsky wheat cultivars were obtained from AVEVE (Landen, Belgium). Tremie wheat was obtained from Dingemans (Stabroek, Belgium). Flaked cereals (barley, wheat, rye, buckwheat, oat, corn, rice, millet) were obtained from Brouwland (Beverlo, Belgium). Specialty malts were obtained from Weyermann Malzfabrik (Bamberg, Germany). Three coloured malts [Vienna (6-8 EBC), Munich (15-20 EBC), Melanoidin (60-80 EBC)], two caramelised malts [Caramunich (110-130 EBC), Caraaroma (300-400 EBC)] and two roasted barley malts [Carafa (1000-1200 EBC) and Carafa special (1330-1500 EBC)] were used.

V.2.2. Laboratory-scale mashing

Unless otherwise specified, standard laboratory Congress wort was produced according to the procedure described in paragraph IV.2.2. After adjusting the beaker content to 450 g, samples were centrifuged (3000 rpm; 5 min) and frozen at -18 °C until analysis.

V.2.3. Total alkali-extractable FA content in wort

Total alkali-extractable FA content in wort was determined according to the procedure described in paragraph III.2.2. using 5.0 mL instead of beer.

V.2.4. Quantification of FA and 4VG in wort

Quantification of FA and 4VG in wort was performed by HPLC-ECD as described in chapter II.

V.2.5. Measurement of endoxylanase (EC 3.2.1.8) activity

Endoxylanase activity assays of wort were performed according to the procedure described in paragraph IV.2.6. using 1.0 mL wort instead of barley malt extract and by incubation at 50 °C for 2 h. The activity is expressed as ΔA_{590} per gram dry malt and per hour incubation.

V.2.6. Measurement of β-D-xylosidase (EC 3.2.1.37) and α-L-arabinofuranosidase (EC 3.2.1.55) activity

Xylosidase and arabinofuranosidase activity assays of wort were performed according to the procedure described in paragraph IV.2.7. using 50 µL of wort instead of barley malt extract. Activities were expressed as EU per gram dry malt. One EU was defined as the amount of enzyme that released 1 µmol *p*-nitrophenol from the substrate per minute at 40 °C and pH 6.0.

V.2.7. Measurement of cinnamoyl esterase (EC 3.1.1.73) activity

Cinnamoyl esterase activity assays of wort were performed according to the procedure described in paragraph IV.2.8. using 1.2 mL of wort instead of barley malt extract.For the determination of the cinnamoyl esterase activity of extracts of specialty malts and wheat samples, the spectrophotometric method could not be used due to the interference of coloured and hazy samples, respectively. Cinnamoyl esterase activities of these samples were assessed against the methyl ester of FA (MFA) with HPLC-ECD analysis as described in paragraph IV.2.8. All extracts were tested both separately (3.0 mL of cereal extract) and in combination

with the barley pilsner malt extract (1.5 mL barley pilsner malt extract + 1.5 mL wheat or specialty malt extract). Activities were expressed as EU per gram malt. One EU was defined as the amount of enzyme that released 1 µmol HCA's from the substrate per minute at 30 °C and pH 6.0.

V.2.8. Effect of the mashing-in temperature, time and pH on FA release

To study the effect of the mashing-in temperature, worts were produced according to the standard Congress wort procedure. Instead of the regular temperature profile, isothermal mashing-in temperatures were set at 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75 and 80 °C. For the determination of free and total alkali-extractable FA contents, samples were taken after 60 min. Samples were immediately cooled by adding 200 mL of ice cold water and 5 mL of phosphoric acid (85 %) was added to stop enzyme activity. After adjusting the beaker content to 450 g, samples were centrifuged (3000 rpm; 5 min) and frozen at -18 °C until analysis. To analyse the content of water-extracted FA, the same procedure was applied but before mashing-in the ground barley malt samples were placed in an oven at 130 °C for 5 h according to Debyser *et al.* (1997c) to eliminate enzyme activity. For the determination of enzyme activities, samples were taken after 10, 20, 30, 40, 60, 80, 100 and 120 min and during sampling, the addition of phosphoric acid was omitted.

At the optimum mashing-in temperature (40 °C), the effect of mashing-in time and pH on free and esterbound FA release was studied. The effect of the mashing-pH was investigated by adjusting the pH of the mash to 4.3, 4.6, 4.9, 5.2, 5.5, 5.8, 6.1 and 6.4 by the addition of adequate amounts of 1 N H_2SO_4 or NaOH. The mashes were kept at 40 °C for 60 min. For the effect of the mashing-in time, the temperature was held at 40 °C for 15 min, 30 min, 1 h, 2 h, 3 h, 4 h and 5 h.

V.2.9. Variation of stirring regime, grist coarseness and mash thickness

To study the effect of the stirring regime on FA release during brewing, laboratory Congress worts were prepared according to the standard procedure. The effect of continuous stirring at 100 and 200 rpm; intermittent stirring at 10 min intervals at 100 and 200 rpm; and no stirring were investigated. The effect of the grist coarseness was evaluated by adjusting the distance between the grinding discs to 0.1, 0.6, 1.2 and 1.9 mm. To study the effect of the mash thickness, the amount of finely ground grist used for mashing-in was set at 45.2, 58.5, 71.9,

85.2, 98.5, 111.9 and 125.2 g (corresponding with final wort densities of 8, 10, 12, 14, 16, 18 and 20 °P).

V.2.10. Addition of wheat, flaked cereal adjuncts and specialty malts

Wort was prepared according to the Congress wort standard procedure replacing 50 % (25.0 g) of pilsner barley malt with finely ground wheat, cereal flakes or specialty malt. Wort samples were filtered over a folded filter (MN 614 ¼ 32 cm diameter, Macherey-Nagel GmbH).

V.2.11. Wort filtration

Wort was prepared according to the Congress wort standard procedure. To assure a good wort run-off, the distance between the grinding discs was set at 1.0 mm and the mashing-off temperature was set at 78 °C. After mashing, the beaker content was adjusted to 450 g with water at 78 °C and the mash was transferred to filtration beakers with perforated bottom plates. During filtration, temperature was kept at 78 °C. After initial mash settling, filtration was started. The first 100 mL was transferred back to the filtration cups. Wort was allowed to run-off until the cake was nearly dry before sparging liquor (200 mL; 78°C) was added. Filtration fractions were collected every 50-100 mL.

V.2.12. Wort boiling and hop addition

Standard laboratory Congress wort preparations were performed according to Analytica-EBC (EBC Analytica, 1998, method 4.5.1). Wort (200.0 g) was heated in a glycerol bath under reflux to prevent evaporation of volatile compounds at 80, 90 and 100 °C. Samples were taken after 0, 30, 60 and 120 min and cooled down rapidly in an ice bath. The influence of the addition of hop flowers and hop pellets on the free FA and 4VG concentration in boiled wort was evaluated by the addition of hop corresponding with a final bitterness of 20 and 40 EBU at the start of the wort boiling process.

V.2.13. Pilot-scale wort brewing (infusion mashing)

Pilsner malt (103 kg) was ground with a single roller malt mill and mashed-in with 3.3 hL water at 45 °C. The mashing-in pH was adjusted to 5.6 with lactic acid (PURAC FCC, VOPAK, Brussels, Belgium). After 15 min, the temperature was raised (1 °C/min) to 78.5 °C with a 10 min hold at 52.5 °C, a 40 min hold at 62 °C and a 15 min hold at 72 °C. The mash was transferred to a classic lauter tun with perforated bottom plate for wort separation. The

spent grains were washed with 2 hL sparging water. The sweet wort was collected in the boiling kettle and heated to boiling temperature by steam injection. The pH was adjusted to 5.3 by the addition of lactic acid. The wort was boiled for 90 min. After 30 min, hop pellets were added to achieve a final bitterness of 20 EBU and after 60 min of wort boiling, additional sugar (1 °P sucrose) was added into the boiling kettle. After boiling, the bitter wort was transferred to the whirlpool for clarification (20 min). Finally, 5 hL wort of 14 °P was obtained. Duplicate samples were taken after 15 min at 45 °C, 10 min at 52.5 °C, 0 min at 62 °C, 40 min at 62 °C, 10 min at 72 °C, mashing-off at 78.5 °C, at the start of wort filtration, during wort filtration before the addition of sparging liquor, at the start of the wort boiling process, during the wort boiling process before the addition of hops, at the end of the wort boiling process and at the end of the whirlpool holding time.

V.2.14. Statistical Analysis

All analyses were carried out at least in duplicate (true replicates) except for the pilot-scale brewery experiment for which duplicate samples were taken at each sampling point. Results are represented as mean ± standard deviation and considered significantly different at a 95 % confidence level (*t*-test statistic; $p < 0.05$).

V.3. Results and Discussion

V.3.1. Effect of mashing-in temperature, time and pH on FA release

The effect of the mashing-in temperature on the release of **free** FA from malt with and without enzyme activity is shown in figure V.1.. FA released from malt without enzyme activity corresponded with the amount that is water-extracted; while FA released from normal malt corresponded with FA that is either water-extracted or enzymatically solubilised by cinnamoyl esterase activity. A clear difference in temperature dependence between the release of water-extracted and enzymatically solubilised phenolic acids was observed. The amount of water-extracted FA was independent of the mashing-in temperature within the observed temperature range. The amount of water-extracted FA corresponded with the amount of FA present in free form in barley malt. This probably originated from cinnamoyl esterase activity during barley germination and the first stages of the green malt kilning. If

FA would be released by additional chemical or thermal hydrolysis during mashing, the water-extraction of FA would increase with higher temperatures. Maximal release of FA from malt with enzyme activity occurred at 40 °C. This is comparable to the 43 °C reported by Narziβ *et al.* (1990) and may explain the elevated concentrations of 4VG reported in beers with an intensified protein rest like Weizenbeers (Back *et al.*, 2000). The curvature of the FA release is typical for an enzyme-catalysed reaction. At 65 °C, almost no FA is being released enzymatically and FA levels in wort drop off to the amount of FA already present in unbound form in malt. McMurrough *et al.* (1996) also showed that increasing the mashing-in temperature from 45 to 65 °C the level of free FA into the wort was significantly decreased.

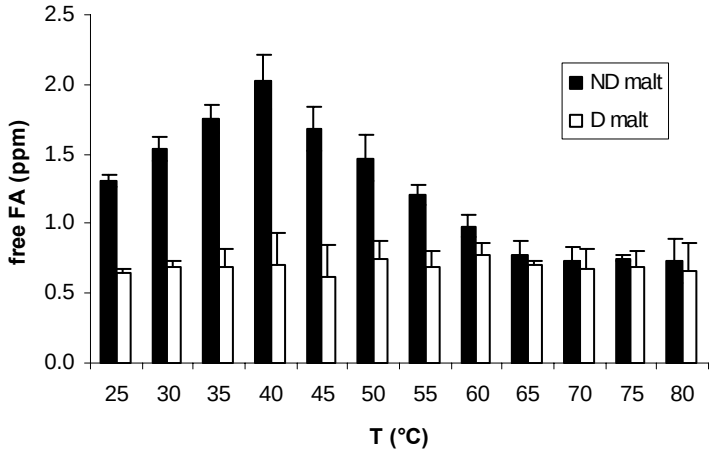


Figure V.1. *Effect of mashing-in temperature on the release of free FA from malt with ("not denaturated" - ND) and without ("denaturated" - D) enzyme activity during wort production*

The effect of the mashing-in temperature on the release of **total** alkali-extractable FA from malt with and without enzyme activity is shown in figure V.2. The amount of total alkali-extractable FA can be considered a measure of the AX level in wort. Hence, the amount of total alkali-extractable FA released from malt without enzyme activity is a measure of the amount of AX being water-extracted from barley malt during wort production while the amount of total alkali-extractable FA from malt with enzyme activity is a measure of the amount of AX being either water-extracted or enzymatically solubilised by the concerted action of AX-degrading enzymes. At temperatures < 65 °C, the amount of total alkali-extractable FA in wort derived from malt with enzyme activity exceeded the amount in wort derived from malt without enzyme activity indicating an enzymatic solubilisation of insoluble AX to water-soluble forms. At temperatures ≥ 65°C, no significant differences could be found between both wort samples. Hence, no enzymatic solubilisation of AX occurred at

these temperatures. In contrast to the amount of water-extracted free FA, the amount of water-extracted total alkali-extractable FA did increase with temperature. Due to the polymeric structure of the AX molecules, their solubility increases with increasing temperatures (Li *et al.*, 2005). At each temperature, most of the AX present in wort originated from the water-extractable part of the malt. Relatively little solubilisation occurred during mashing as was previously shown by Debyser *et al.* (1997a).

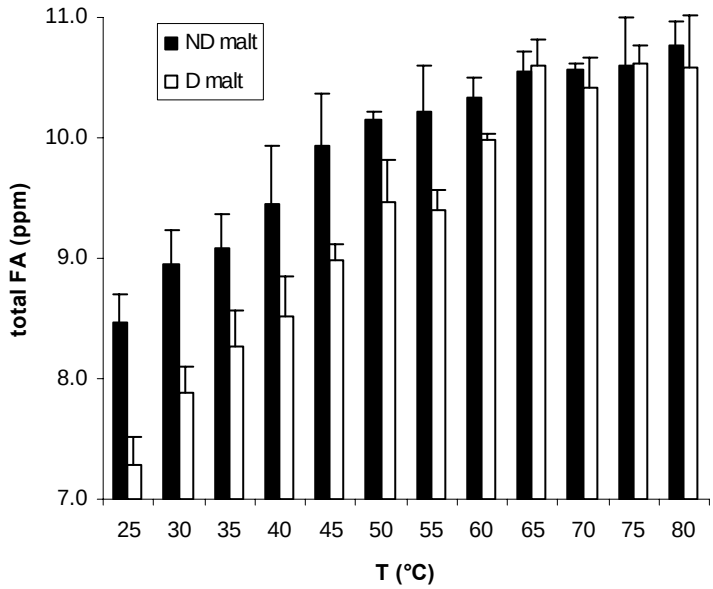


Figure V.2. *Effect of mashing-in temperature on the release of total alkali-extractable FA from malt with ("not denaturated" - ND) and without ("denaturated" - D) enzyme activity during wort production*

At the optimal mashing-in temperature (40 °C), the effect of mashing-in pH on free and esterbound FA release from malt with and without enzyme activity was studied. The effect of the mashing-pH was investigated by adjusting the pH of the mash to 4.3, 4.6, 4.9, 5.2, 5.5, 5.8, 6.1 and 6.4. The mashes were kept at 40 °C for 60 min. Initial pH-values and values after mashing-in with malt with and without enzyme activity are shown in table V.1.

TableV.1. *Initial pH-values and values after mashing-in for 60 min at 40 °C with malt with (ND-malt) and without (D-malt) enzyme activity*

$pH_{initial}$	pH_{end} ND-malt	pH_{end} D-malt
4.30	4.69	4.89
4.60	5.09	5.09
4.90	5.46	5.33
5.20	5.69	5.44
5.50	5.89	5.57
5.80	6.03	5.97
6.10	6.17	6.20
6.40	6.32	6.44

The release of free FA from malt with and without enzyme activity during wort production at different pH's is shown in figure V.3.

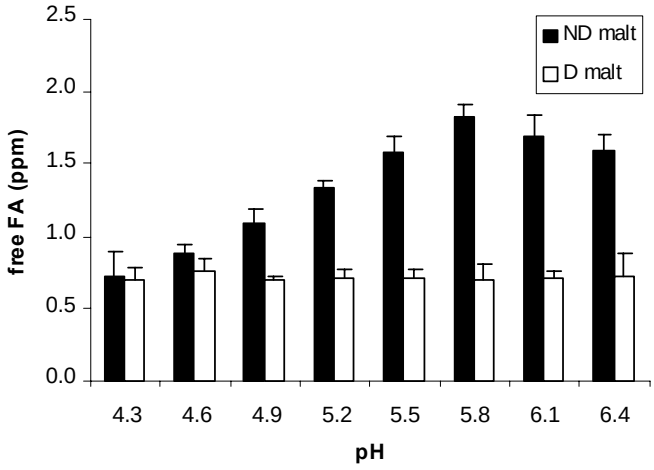


Figure V.3. *Effect of mashing-in pH on the release of free FA from malt with ("not denaturated" - ND) and without ("denaturated" - D) enzyme activity during wort production at 40 °C*

The release of water-extractable FA from malt without enzyme activity was independent of the pH. Hence, within the observed pH range, no acid hydrolysis of the esterbond between FA and AX occurred. At pH < 4.6, no significant differences could be found between the amount of FA released from malt without enzyme activity and normal malt. Hence, no significant enzymatic release of FA by cinnamoyl esterase activity did occur at low pH. This is consistent with the results from Humberstone *et al.* (2000) who found that at pH 4 the cinnamoyl esterase enzyme in extracts from malted barley is inactive. At pH > 4.6, significant enzymatic hydrolysis of esterbound FA occured with an optimal FA release at pH 5.8. Narziβ *et al.* (1990) found a slightly higher optimal pH for FA release of 6.0. The results were consistent with those found by Back *et al.* (2000) who found that mash acidification leads to a decrease in FA release during wort production. Within the observed pH range, no effect was observed on the amount of total alkali-extractable FA either from malt with or without enzyme activity (results not shown). Slade *et al.* (1989) found that the barley xylan endohydrolase maintains up to 75 % of its maximal activity over a broad pH range (4.5 to 7.2). This relative stability of the endoxylanase can explain the independence of total alkali-extractable FA during wort production over the observed pH range (4.3-6.4).

The effect of the mashing-in time was evaluated at 40 °C. Samples were taken after 15 min, 30 min, 1 h, 2 h, 3 h, 4 h and 5h. The results of the influence of the mashing-in time on the release of free FA from malted barley are shown in figure V.4.

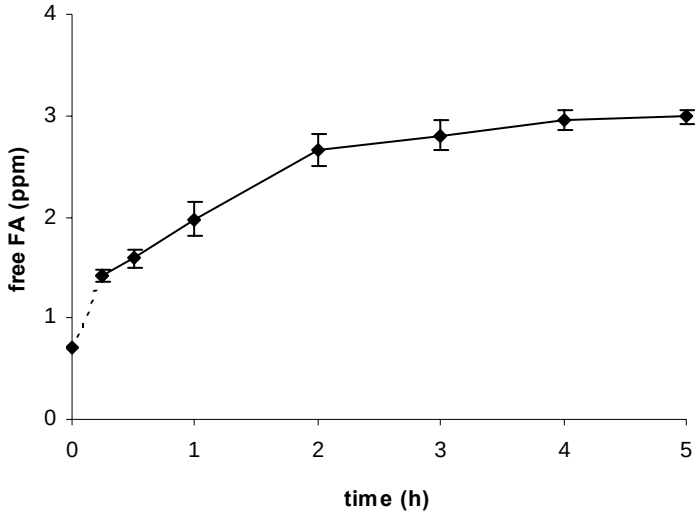


Figure V.4. *Effect of the mashing-in time on the release of free FA from malt with enzyme activity during wort production at 40 °C*

At the start of mashing-in, a rapid increase in FA concentration occurred. After 15 min already 1.41 ppm FA was released into the wort in free form half of which was water-extracted (0.7 ppm) while the other half was enzymatically released. Taken into account that the flavour threshold of 4VG in blond specialty beers is 370 ppb (chapter III), this gave rise to a wort with a 4VG potential of 3 flavour units (FU). After 1 h, free FA further increased to 2.0 ppm and further incubation lead to a final free FA content of 3.0 ppm in the wort. However, taken into account the esterbound FA content, no more than 23 % of the total alkali-extractable FA in wort was hydrolysed.

V.3.2. AX-degrading enzyme activities during wort production

The activity of 4 AX-hydrolysing enzymes during iso-thermal mashing is represented in figure V.5. The main-chain hydrolysing enzyme endoxylanase remained its optimal activity up to 45 °C but the activity rapidly decreased at higher mash temperatures. At 65 °C and 70 °C, almost no endoxylanase activity could be detected. This explains why, at temperatures exceeding 65 °C, no additional AX were solubilised in wort made from normal malt compared to the wort made from malt with denaturated enzymes (figure V.2). The observed temperature dependence of the endoxylanase enzyme during mashing was highly consistent with that found Li *et al.* (2005).

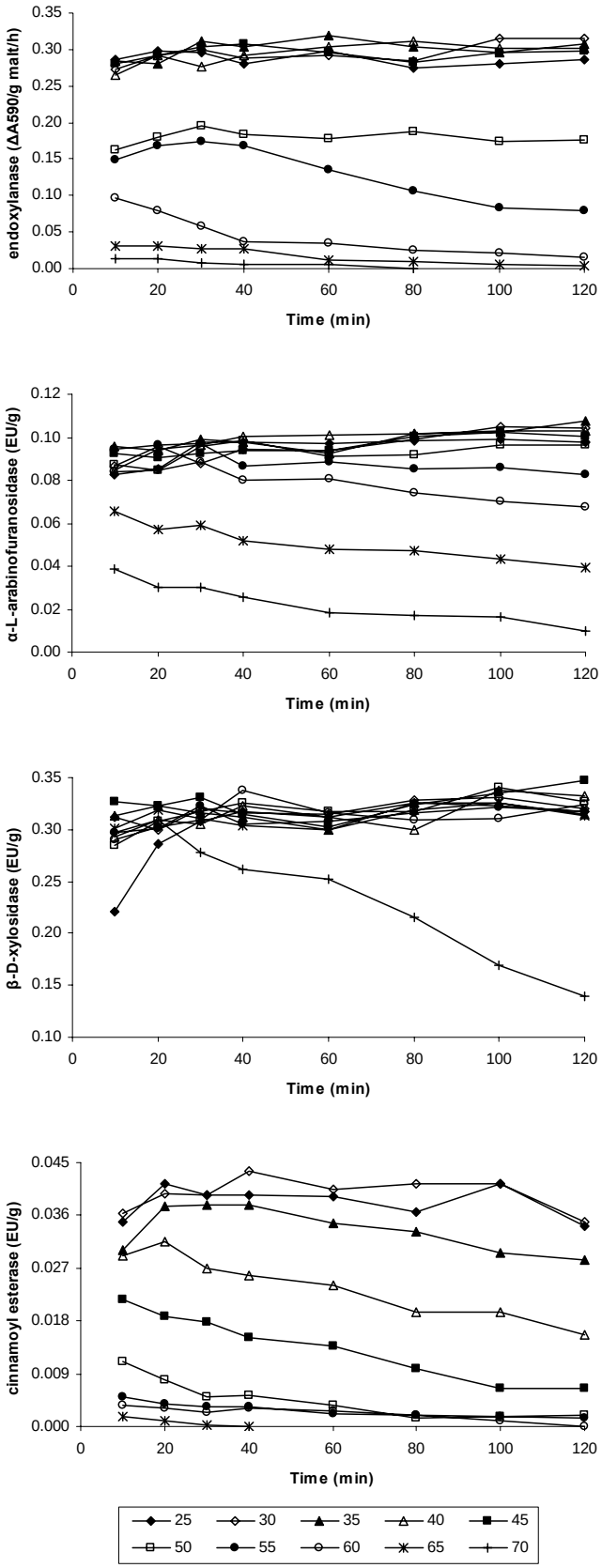


Figure V.5. *AX-hydrolysing activities in isothermal mashes: endoxylanase activity, α-L-arabinofuranosidase activity, β-D-xylosidase activity and cinnamoyl esterase activity (from top to bottom)*

The α-L-arabinofuranosidase was optimally active up to 50 °C. At temperatures exceeding 50 °C, activity decreased with increasing temperatures. Of the four enzymes examined, the β-D-xylosidase enzyme was the least sensitive to elevated temperatures. Only at 70 °C, a decrease in enzyme activity was observed. Similar findings were observed by Debyser *et al.* (1998) during brewing by the infusion method, although they suggested an optimal temperature of the endoxylanase enzyme at 50 °C. At 25 °C, the β-D-xylosidase activity increased remarkably during the first 30 min of mashing due to the solubilisation of the enzyme from the malt. At low temperatures, a similar increase, although less pronounced, in endoxylanase and cinnamoyl esterase activity was found. The optimal temperature of cinnamoyl esterase activity was 30 °C. After 60 min at 40 °C, the activity of the cinnamoyl esterase enzyme was only 40 % of the maximal activity. This corresponded with the results obtained by Humberstone *et al.* (2000) for cinnamoyl esterase activity in an extract of barley malt and feruloyl glycerol as substrate. At higher temperatures, the cinnamoyl esterase was rapidly denaturated and at 65 and 70 °C no activity could be detected. This explains why no difference was found between the FA release from malt with and without enzyme activity at temperatures exceeding 60 °C (figure V.1). Remarkably, the temperature of maximal FA release (40 °C) does not correspond with the optimal temperature of the cinnamoyl esterase activity (30 °C). The discrepancy between both temperatures can be explained by the synergy between the cinnamoyl esterase enzyme and other AX-degrading enzymes like the endoxylanase activity in the release of FA during brewing as was shown in chapter IV. The concerted action of the AX-hydrolysing enzymes does not only increase the amount of feruloylated AX in wort. They also have a depolymerising activity decreasing the molecular weight of the AX in solution. This renders them more accessible and makes them more susceptible to cinnamoyl esterase enzymes. Probably this depolymerising activity mechanism is more important than the solubilising activity of the AX-hydrolysing enzymes since most of the alkali-extractable FA present in wort is bound to WEAX (figure V.2). Since the optimal activity of the AX-hydrolysing enzymes was higher than that of the cinnamoyl esterase (figure V.5), the temperature of optimal FA release during mashing is shifted up towards the temperature of optimal activities of other AX-degrading enzymes.

V.3.3. Variation of stirring regime, grist coarseness and mash thickness

The effect of the stirring regime on the release of free FA from malt during wort production is presented in table V.2. Continuously stirred wort at 200 rpm corresponds with the Congress wort brewing procedure.

Table V.2. *The effect of the stirring regime on the release of free FA from malt during wort production*

Stirring regime	Free FA (ppm)
No stirring	1.45 ± 0.02
Continuous stirring – 100 rpm	1.83 ± 0.02
Continuous stirring – 200 rpm	2.10 ± 0.02
Intermittent stirring – 100 rpm	1.74 ± 0.01
Intermittent stirring – 200 rpm	1.93 ± 0.07

When the mash was stirred during brewing, significantly more FA was released into the wort compared to the unstirred wort. De-intensifying the stirring regime by stirring at 100 rpm instead of 200 rpm or intermittent stirring instead of continuous stirring decreased the amount of FA released from the malt. When the wort was continuously stirred at 200 rpm, 45 % more FA was released compared to the unstirred wort. Similarly, Li *et al.* (2005) found that the AX content in a continuously stirred mash almost doubled compared to the AX content of an unstirred mash due to the effect of physical and mechanical forces on AX solubilisation. An increased in the extraction and solubilisation of AX from the malt will lead to significant higher esterbound FA concentrations in the wort. Hence, more substrate is available for the cinnamoyl esterase enzyme, leading to higher free FA levels in wort.

The effect of the grist coarseness on the release of free FA from malt during wort production was evaluated by adjusting the distance between the grinding discs to 0.1, 0.6, 1.2 and 1.9 mm. Results are represented in table V.3.

Table V.3. *The effect of the grist coarseness on the release of free FA from malt during wort production*

Distance between grinding discs	Free FA (ppm)
0.1 mm	2.04 ± 0.01
0.6 mm	1.82 ± 0.02
1.2 mm	1.75 ± 0.02
1.9 mm	1.58 ± 0.04

The coarseness of the grist had a remarked effect on the release of free FA during brewing. More FA was released from fine grist than from coarse grist. The same effect of the grist coarseness on the solubilisation of AX during brewing was observed (Li *et al.*, 2005). The coarse grist limited AX solubilisation, resulting in low AX concentrations in the wort. This decreased wort AX content lead to significant lower esterbound FA concentrations limiting the substrate availability for the cinnamoyl esterase enzyme. Practically, brewhouse conversion from a classic lauter tun to a new high-pressure membrane mash filter, generally

leads to the use of more fine grist since no spent grain filtration bed is needed for wort separation. This may lead to a higher phenolic aroma potential of the wort.

The effect of the mash thickness on the release of free FA during wort production is shown in figure V.6.

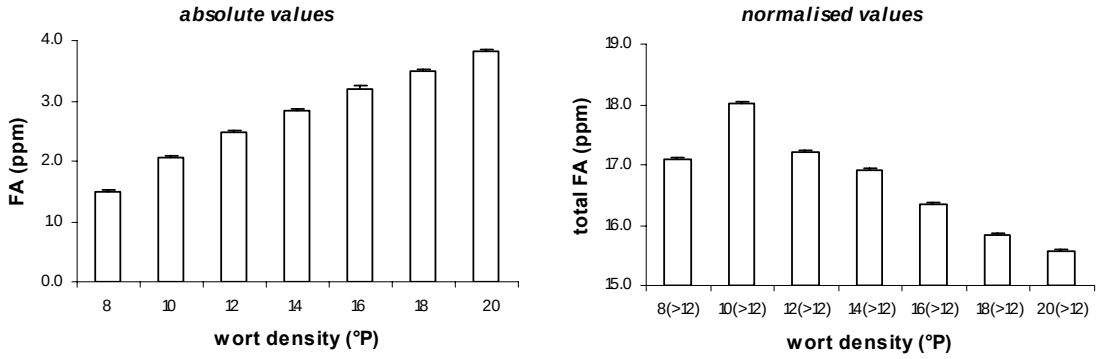


Figure V.6. *Effect of the mash thickness (°P) on the release of free FA (ppm) during wort production: absolute FA concentrations (left) and normalised FA concentrations* vs. *12 °P (right)*

Clearly, when the mash became denser, the absolute concentration of free FA in wort increased. Increasing the amount of malt will increase both the water-extracted and the enzymatically solubilised amount of FA present in wort. However, when the FA concentration was normalised for the amount of malt used for the wort of 12 °P, it can be seen that relatively more FA was solubilised in worts of medium density. Both in more diluted worts and in high gravity worts, relatively less FA is released into the wort. The parabolic curvature of the effect of the mash thickness on the FA release can be explained by two counteracting mechanisms. Generally, enzymes are appreciably more stable in thicker mashes (with higher grist:water ratios) (Briggs, 1998). This may protect enzymes from thermal inactivation in more dense worts. However, in thick mashes, the water phase becomes more concentrated. This increased viscosity may limit the mobility of the substrate in high gravity worts and the solubilisation of AX (Li *et al.*, 2005). Practically, brewing at high gravity followed by wort dilution will lead to wort with less phenolic aroma potential than mashing-in at the corresponding medium gravity.

V.3.4. Addition of wheat, flaked cereal adjuncts and specialty malts

The influence of the addition of 11 wheat varieties during brewing on the level of free FA in Congress wort is shown in figure V.7.

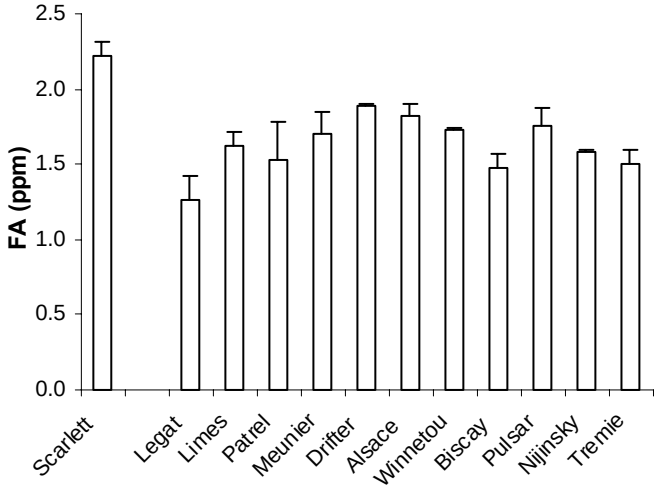


Figure V.7. *Influence of the addition of different wheat varieties during brewing on free FA concentration (ppm) in Congress wort (50 % wheat – 50 % Scarlett pilsner malt)*

Significant differences were found between worts produced with different wheat cultivars. Moreover, free FA levels were consistently lower in wort with 50 % wheat and 50 % pilsner malt than the concentration of free FA in the reference pilsner wort (Scarlett wort). Worts produced with 50 % unmalted wheat contained 15 to 43 % less free FA than the corresponding wort with 100 % malted barley. To test if the decrease of free FA could be attributed to a lower cinnamoyl esterase activity of wheat, the esterase activities of Scarlett malt and wheat (Drifter, Legat and Tremie) extracts were tested both separately and in combination with the barley malt pilsner extract (v/v % 50/50). Esterase activities were 1.26 ± 0.12 EU/g, 2.33 ± 0.16 EU/g, 3.06 ± 0.16 EU/g and 2.30 ± 0.15 EU/g for Scarlett, Tremie, Drifter and Legat, respectively. Hence, the three wheat extracts had a higher cinnamoyl esterase activity than the Scarlett malt. Raw cereals containing more esterase activity than malted cereals can be explained by the decrease of esterase activity during germination (Sancho *et al.*, 2001) and its heat sensitivity (Humberstone and Briggs, 2000; Sun *et al.*, 2005) causing a further decrease during kilning. The esterase activities of the combined extracts (1.88 ± 0.23 EU/g, 1.82 ± 0.34 EU/g and 2.12 ± 0.16 EU/g for Tremie, Drifter and Legat, respectively) were not significantly different from the theoretical values (½ esterase activity of barley malt + ½ esterase activity of wheat) indicating that the barley malt cinnamoyl esterase is not inhibited by wheat extracts. Since worts produced with 50 % wheat had higher cinnamoyl esterase activities than pilsner wort, the lower amount of FA released in wort produced with wheat was certainly not due to a lower esterase activity. Another possible reason for the decrease in free FA was a lower release of water-extracted FA or a

lower alkali-extractable FA content (i.e. cinnamoyl esterase substrate) in worts produced with wheat. However, compared to the pilsner wort (0.64 ppm water-extracted FA and 7.54 ppm alkali-extractable FA) the worts produced with the three wheat varieties did not contain lower levels of water-extracted (0.49 ± 0.17 ppm) or alkali-extractable FA (7.44 ± 0.82 ppm). Since the decreased release of FA in worts produced with 50 % unmalted wheat cannot be explained by a lower cinnamoyl esterase activity or a lower level of cinnamoyl esterase substrate, other factors need to be taken into consideration. As already postulated, wort endoxylanase activity and AX molecular weight distribution may have a profound effect on the release of FA during mashing. Since the endoxylanase activity has only minor effects on the final AX content in wort, the alkali-extractable FA content in wort will depend mainly on the WEAX content of the starting material. WEAX of barley malt have a much lower molecular weight than those from wheat: 38 kDa (Debyser *et al.*, 1997b) compared to 400 kDa (Cleemput *et al.*, 1995b), respectively. Hence barley malt WEAX may be better substrates for cinnamoyl esterases than wheat WEAX due to less steric hindrance. Moreover, endoxylanase activity of worts produced with unmalted wheat have been found to be lower than endoxylanase activity of worts produced with 100 % barley malt (Debyser *et al.*, 1998). This is probably due to the inhibition of the exogenous microbial xylanolytic system associated with wheat kernels by wheat endogenous endoxylanase inhibitors (Dornez *et al.*, 2006). Apart from differences in endoxylanase activity, wheat and barley malt WEAX also differ in molecular structure. Compared to wheat AX, barley AX appear to be more structurally uniform in which substituted residues are more clustered and separated by regions of unsubstituted xylosyl units. Arabinose substitution along the backbone is not random. Barley AX also have a higher proportion of arabinose substituted at the O-2 position compared to wheat AX (Vietor *et al.*, 1992). In conclusion, it seems that the limited release of FA in worts produced with unmalted wheat was due to the lower extent of AX degradation or differences in the AX structure, rather than a direct inhibition of the esterase activity.

The influence of the addition of specialty malts during brewing was examined by the production of Congress wort with 50 % pilsner barley malt and 50 % specialty malts. Three coloured malts [Vienna (6-8 EBC), Munich (15-20 EBC), Melanoidin (60-80 EBC)], two caramelised malts [Caramunich (110-130 EBC), Caraaroma (300-400 EBC)] and two roasted barley malts [Carafa (1000-1200 EBC) and Carafa special (1330-1500 EBC)] were used. The content of free FA and 4VG in Congress worts is shown in figure V.8.

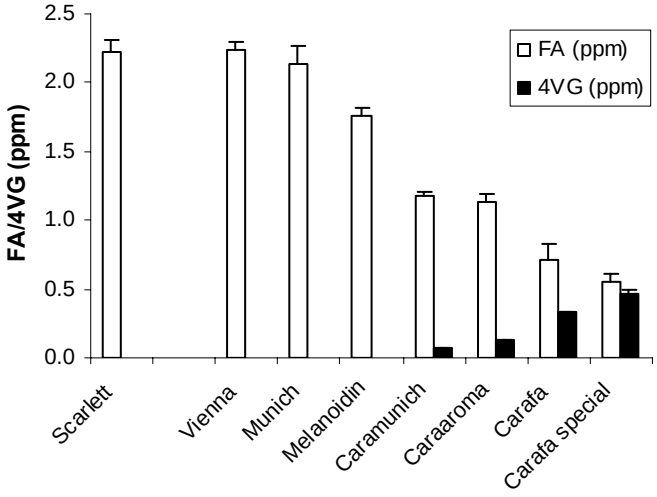


Figure V.8. *Influence of the addition of specialty malts during brewing on free FA en 4VG concentration (ppm) in Congress wort (50 % specialty malt – 50 % Scarlett pilsner malt)*

Worts produced with Vienna and Munich coloured malts did not significantly differ in FA content from the reference pilsner wort. Concerning the other specialty malts, the free FA content in Congress wort decreased with increasing wort colour. In the worts produced with the caramelised and roasted malts, 4VG could also be detected in increasing levels with increasing malt colour. It arises from the thermal fragmentation of free FA in barley grain during the kilning and roasting of these highly coloured malts. Although, the thermal decarboxylation of free FA during the malt production process can only account partly for the lower levels of free FA in worts produced with specialty malts. The worts produced with Carafa and Carafa special even contained less than 50 % of the reference pilsner wort. Cinnamoyl esterase activities of Scarlett malt and 3 specialty malts (Melanoidin, Caramunich and Carafa) extracts were both tested separately and in combination with the barley malt pilsner extract (v/v % 50/50). Esterase activities were 1.26 ± 0.12 EU/g and 0.45 ± 0.13 EU/g for Scarlett and Melanoidin malt, respectively. No cinnamoyl esterase activity could be detected in Caramunich and Carafa malt extracts. Endoxylanase activity will probably also decrease with increasing malt colour due to thermal denaturation. The esterase activities of the combined extracts of Melanoidin and Caramunich (0.83 ± 0.04 EU/g and 0.65 ± 0.09 EU/g, respectively) were not significantly different from the theoretical values (½ esterase activity of barley malt + ½ esterase activity of specialty malt) indicating that the barley malt cinnamoyl esterase was not inhibited by these extracts. However, the cinnamoyl esterase activity of the combined extract of Carafa (0.32 ± 0.06 EU/g) was only half the theoretical value (0.63 ± 0.06 EU/g) indicating that the extract of the roasted malt inhibited the esterase activity of the Scarlett pilsner malt. Since roasted barley or roasted malt generally make up

only a small proportion of the grist, Congress worts were also produced with 5 % specialty malt. The free FA content of these worts did not significantly differ from the reference pilsner wort. 4VG could only be detected in the worts produced with Caramunich and Carafa malt (< 50 ppb). Hence, when used in small amounts, the addition of specialty malts during brewing will not affect wort HCA's and volatile phenol concentrations significantly.

The influence of the addition of flaked cereals during brewing was examined by the production of Congress wort with 50 % pilsner barley malt and 50 % flaked cereals. While in the previous experiments, the release FA and pCA followed parallel trends, the addition of flaked cereals had different effects on the release of FA and pCA. The content of free FA and pCA in Congress worts with 8 different flaked cereals is shown in figure V.9.

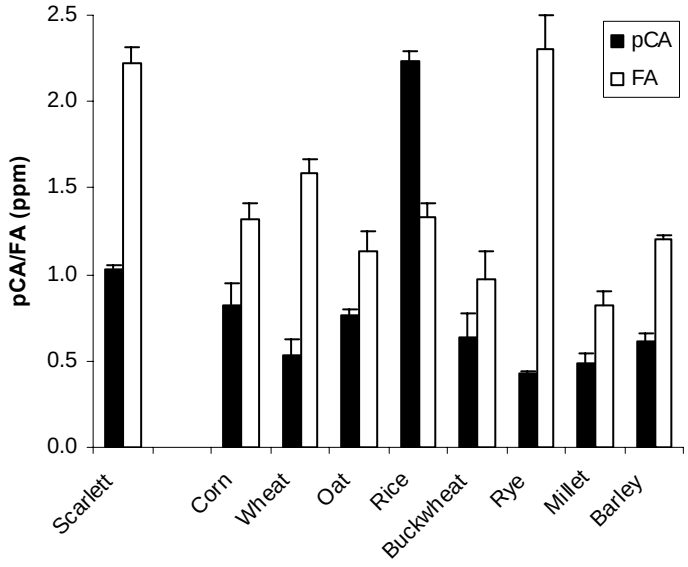


Figure V.9. *Influence of the addition of cereal flaked adjuncts during brewing on free pCA and FA concentration (ppm) in Congress wort (50 % cereal adjunct – 50 % Scarlett pilsner malt)*

Except for the pCA content in wort produced with rice flakes and the FA content in wort produced with rye, the worts produced with cereal adjuncts had significant lower levels of free pCA and FA. During the production process of the flakes, all enzymes are denaturated. The lack of cinnamoyl esterase activity and endoxylanase activity of these adjuncts will generally lead to reduced levels of pCA and FA in wort. The FA content in wort produced with rye flakes was comparable to the FA content in pilsner wort. This was probably due to the high levels of AX reported in rye (Henry, 1987).

V.3.5. Wort filtration

The influence of the separation of wort and spent grains on the content of free FA in wort, was evaluated in laboratory-scale filtration experiments. Grist coarseness and mashing-off temperature during Congress wort production was adapted to assure a good wort run-off during filtration. Free FA concentration at mashing-off, before filtration, was 2.14 ± 0.08 ppm. The duration of the initial mash-settling (0, 15, 30 and 60 min) had no effect on the final free FA concentration in wort after filtration. After the initial run-off, before sparging liquor was added, free FA concentration in the filtered wort was 1.81 ± 0.12 ppm. Hence, a significant amount of free FA was retained in the unwashed spent grain cake. However, washing the spent grains with sparging liquor, a standard procedure to assure maximum recovery of extract, lead to the complete recovery of the free FA initially retained in the spent grain cake. Taking the dilution factor into account, the final free FA concentration in wort was 2.02 ± 0.06 ppm, which did not significantly differ from the free FA concentration before filtration.

V.3.6. Wort boiling and hop addition

The formation of 4VG by thermal decarboxylation of FA in Congress pilsner wort is shown in figure V.10.

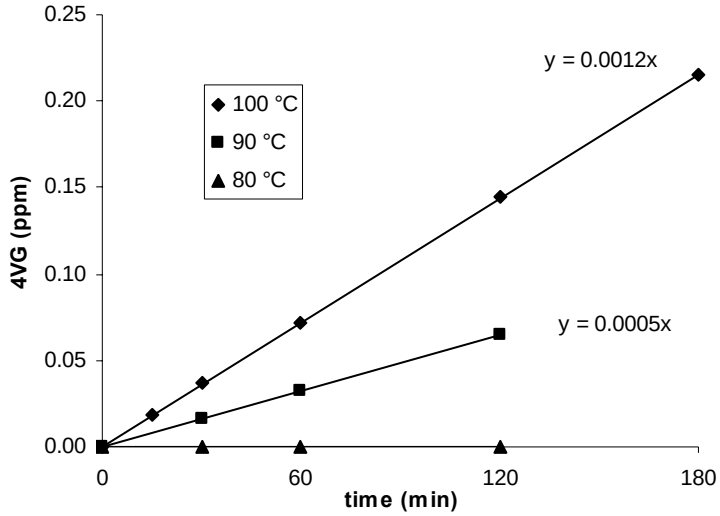


Figure V.10. *Effect of thermal load (80 °C, 90 °C and 100 °C) during wort heating on 4VG formation (ppm) in standard Congress wort with Scarlett malt*

Mashing temperatures (up to 80 °C) were inadequate for the thermal decarboxylation of FA as no 4VG could be detected in unboiled wort. At 90 and 100°C, 4VG concentrations increased with the heating time. Fiddler *et al.* (1967) noticed that, in dry atmosphere, FA is

only degraded starting from 200 °C, indicating that the thermal decarboxylation is greatly enhanced under aqueous reaction conditions as already stated by Coghe *et al.* (2004a). No additional release of free FA or other thermal degradation products of FA (vanillin, acetovanillon of vanillic acid) could be detected. At 90 and 100 °C, a linear relationship was found between the concentration of 4VG and the time of the thermal treatment. Most of the degradation reactions have been found to be first order, the rate of the formation of degradation products of a component being directly proportional to its concentration. However, when the concentration of the reactant is fairly high, it may remain relatively constant over heating time implying that the rate of the formation of degradation products is also constant over time (Marcotte *et al.*, 1998). The reaction mechanism of the thermal degradation of FA is pseudo-zero-order and the 4VG concentration in wort may be expressed as

$$[4VG]_t = kt + [4VG]_0$$

with $[4VG]_t$ being the final 4VG concentration in the wort after heating and $[4VG]_0$ the initial 4VG content in the unheated wort, which in this case was zero but may be different from zero when dark specialty malts are used in the grist. The rate constants k (calculated as the slope of the regression line forced through zero) were determined to be 0.0005 and 0.0012 ppm x min for 90 °C and 100 °C, respectively. The activation energy was calculated from the logarithmic form of the Arrhenius equation ($\ln k = \ln A - Ea/R(1/T)$). Out of the natural log of the rate constants against the reciprocal of the temperature, an activation energy Ea of 103 kJ/mol and a frequency factor A of 4.32×10^{11} were calculated. The change in reaction rate constant accompanying a 10 °C change in temperature, expressed as Q10 ($k_{100°C}/k_{90°C}$), is 2.4, which is in the order of 2 as expected for a chemical reaction. Concerning the wort pH and mash acidification, no effect was found on the formation of 4VG within the investigated pH range (pH 5-7) (results not shown).

From the rate constant at 100 °C and the flavour thresholds of 4VG determined in pilsner beer, blond beer and white beer (chapter III), the wort boiling times that would give rise to 4VG concentrations above the flavour threshold can be calculated. The boiling times were determined to be $1h^3/_4$, $2h^3/_4$ and 5 h in pilsner beer, white beer and blond beer, respectively. Hence for wheat beers and blond specialty beers, thermal degradation of FA will be of minor importance for the phenolic character of beer. Especially in blond specialty beers, the high 4VG concentration observed cannot be explained by the thermal degradation of FA alone.

However, in pilsner beer, the combined time of wort boiling, transfer, whirlpool and pasteurisation times can give rise to 4VG concentrations above the flavour threshold as was observed in the analysed commercial pilsner beers described in chapter III. Hop addition lead to a net increase in free FA content in wort (+7.6 % 20 EBU pellets, +6.6 % 20 EBU flowers, +10.3 % 40 EBU pellets and +11.1 % 40 EBU flowers). No significant difference was found between the addition of hop pellets or hop flowers.

V.3.7. Pilot-scale wort production by infusion mashing method: a case study

The release and the evolution of free FA during wort production (infusion mashing) in a pilot-scale brewery (5 hL) were investigated by multiple sampling during the mashing process, filtration, wort boiling and whirlpool holding. The results are represented in figure V.11.

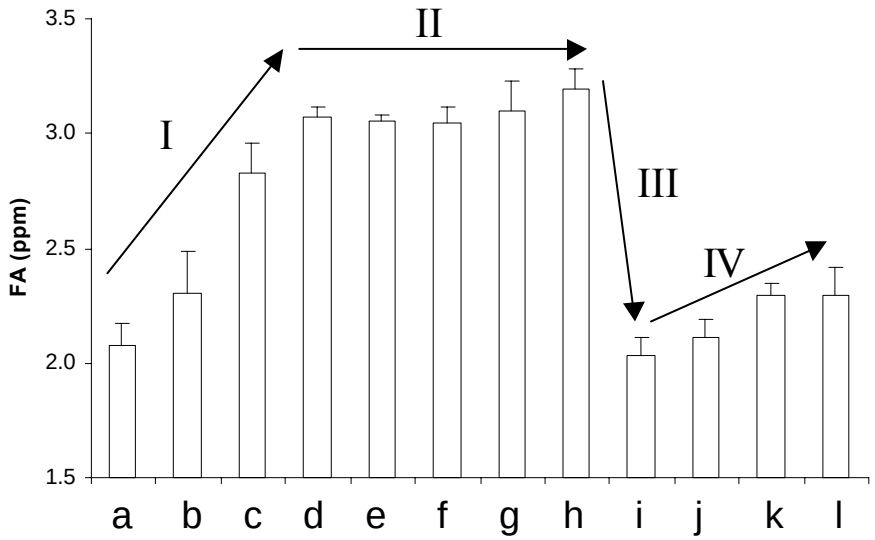


Figure V.11. *Release and evolution of free FA (ppm) during wort production: a case study (a) 45 °C 15 min; (b) 52.5 °C 10 min; (c) 62 °C 0 min; (d) 62 °C 40 min; (e) 72 °C 10 min; (f) 78.5 °C mashing-off; (g) start filtration; (h) during filtration before sparging; (i) end of filtration – start wort boiling; (j) during wort boiling – before hopping; (k) end wort boiling; (l) whirlpool end*

Four distinct parts can be differentiated during the course of the wort production process. From the start of mashing-in until the end of the holding time at 62 °C (I), the FA concentration increased continuously due to the extraction of free malt FA and the enzymatic solubilisation of esterbound FA at temperatures < 65 °C. The highest amount of FA was released during the first 15 min of mashing. After the initial increase, the wort FA content levelled-off and remained constant (II) during the mashing-off phase, the transfer to the lauter tun, the mash settling and the start of the wort filtration until the sparging of the spent grains.

When the temperature exceeded 62 °C, the cinnamoyl esterase enzyme was denaturated. Since no thermal or chemical hydrolysis of FA occurred at higher temperatures (72 and 78.5 °C), no additional FA was released into the wort. During the washing of the spent grains, a decrease in wort FA content was seen due to the dilution of the wort with the sparging liquor (III). The drop in the FA concentration corresponded with the wort dilution factor. Hence, no significant amount of free FA remained in the filter bed after washing.

During wort boiling, the free wort FA concentration increased with 10 % (IV). This net increase was the result of several factors. During wort boiling, thermal decarboxylation of FA will lead to the formation of 4VG. At the end of the boiling process, 0.14 ppm 4VG was found in the wort. This thermal decarboxylation caused the wort FA concentration to diminish by 9 %. However, during wort boiling, the wort volume will decrease by 7-8 % due to evaporation. This will cause an apparent increase in FA content. Finally, the addition of hop pellets will cause a real increase in wort FA content by 7.6-11.1 %. Taking into account these three factors, a net increase of the wort FA content during wort boiling will occur. The reassociation or coprecipitation of free FA with AX, polyphenols or proteins was negligible. Otherwise, no net increase in free FA content would occur during pilot-scale wort boiling.

V.4. CONCLUSION

During mashing, FA is both water-extracted and enzymatically solubilised by cinnamoyl esterases. A clear difference in temperature and pH dependence between the release of the water-extracted and the enzymatically hydrolysed fraction was found. In contrast to the water-extracted fraction, the hydrolysis of esterbound FA is subject to close technological control. An optimal temperature of 40 °C and an optimal pH of 5.8 were found. Also the mash thickness, the grist coarseness and composition, and the stirring regime had a profound effect on the release of FA during mashing. The T,t-dependence of AX degrading enzymes was correlated with free and esterbound FA release during mashing. Finally, a pilot-scale (5 hL) wort production process was conducted to validate the results of the laboratory-scale mashing experiments. Concerning thermal decarboxylation, in pilsner beer, the combined time of wort boiling, transfer, whirlpool and pasteurisation times can give rise to the 4VG

concentrations observed in the survey (chapter III). However for wheat beers and blond specialty beers, thermal degradation of FA was found to be of minor importance for the phenolic character of beer.

CHAPTER VI

CHARACTERISATION OF *SACCHAROMYCES CEREVISIAE* PHENYLACRYLIC ACID DECARBOXYLASE (PAD1) ACTIVITY [4]

In this chapter, the role of brewing yeast strains on the formation of volatile phenols during fermentation was investigated. A survey among S. cerevisiae *yeast strains was conducted and showed a high incidence of the Pad1 phenotype. The enzymatic decarboxylation of hydroxycinnamic acids by phenylacrylic acid decarboxylase activity during alcoholic fermentation was studied.*

[4] Part of this chapter is based on the following publication: Vanbeneden, N., Delvaux, F., Delvaux, F.R. Formation of 4-vinyl and 4-ethyl derivatives from hydroxycinnamic acids: occurrence of volatile phenolic flavour compounds in beer and distribution of Pad1-activity among brewing yeasts. *Food Chemistry*, 107(1): 221-230.

VI.1. INTRODUCTION

In the previous chapters, the release of HCA's during wort production was investigated. These HCA's are flavour-inactive and do not contribute to the aroma of beer. To contribute to the odour and taste pallet of specialty beers, they have to be decarboxylated to the corresponding volatile phenols. The contribution of thermal decarboxylation during wort boiling has already been discussed in chapter V. Volatile phenol concentrations formed during high temperature treatments, did not substantially contribute to the phenolic flavour impression of specialty beers. Hence, the high concentrations often encountered in wheat beers, and blond and dark specialty beers must originate from the enzymatic decarboxylation by *Saccharomyces cerevisiae* yeast strains during wort fermentation. In this chapter, the role of brewing yeast strains will be further characterised. A survey among top-fermenting *Saccharomyces cerevisiae* and bottom-fermenting *Saccharomyces pastorianus* yeast strains and *Brettanomyces/Dekkera* spp. was conducted to examine the occurrence of Pad1-phenotype among brewing yeast strains. The substrate specificity of the Pad1 enzyme and the time course of 4VG formation during alcoholic fermentation were studied. The temperature and pH dependence of the Pad1 enzyme activity of yeast crude extract were examined and the effect of the ethanol content and FA concentration on the decarboxylase activity were assessed. The influence of different process parameters during fermentation of wort or YPD (i.e. temperature, pH, pitching rate, FA concentration, the use of specialty malts, the kind of carbon source and the effect of yeast storage) were examined. Bottle refermentation experiments were conducted to test the effect of bottle conditioning on 4VG formation. Finally, two pilot-scale fermentation experiments were set-up to study the effect of top pressure applied during wort fermentation and gain more insight into the difference between top *versus* bottom cropping of yeast.

VI.2. MATERIALS AND METHODS

VI.2.1. Quantification of FA and 4VG in wort

Quantification of FA and 4VG in wort was performed by HPLC-ECD as described in chapter II.

VI.2.2. Screening for Pad1 phenotype

Saccharomyces cerevisiae brewing yeasts and *Brettanomyces/Dekkera* ssp. were screened for Pad1-activity by inoculating single isolated colonies of purified yeast strains into 10 mL YPD-medium (40 g/L glucose, 20 g/L peptone and 10 g/L yeast extract) supplemented with 0.5 mM pCA, FA or SA (from a 1 % w/v stock solution in ethanol) in duplicate. *Saccharomyces cerevisiae* industrial brewing yeast strains were selected from the yeast collection of the Centre for Malting and Brewing Science (CMBS). After semi-anaerobic incubation at 25 °C for 3 days, the supernatant was analysed on the content of volatile phenols by HPLC-ECD.

VI.2.3. Tall tube fermentation experiments

Yeasts were propagated by inoculating single isolated colonies of purified yeast strains into 10 mL YPD medium. After incubation at 25 °C for 48 h, yeast cells were transferred to 150 mL wort (12 °P) and incubated on a rotary shaker at 20 °C for 48 h. Yeast cells were harvested by centrifugation (3000 rpm, 3 min, 4 °C) and resuspended in physiological water (0.9 % NaCl). Yeast cell densities were determined microscopically with a Thoma counting chamber. Standard 12 °P wort (1.8 L) supplemented with 3 °P glucose and 0.1 mM pCA, FA or SA (from a 1 % w/v stock solution in ethanol) was pitched with 5 x 10^6 propagated yeast cells per mL in EBC tall tubes. Incubation temperature during fermentation was set at 20 °C for 10 days. During fermentation, samples were taken daily 10 cm below the fluid surface. Yeast cell densities were determined with a Thoma counting chamber. Samples were analysed for volatile phenol content by HPLC-ECD and for extract content using a density and sound analyser (DSA-4, A. Paar, Graz, Austria) with a SP-1 autosampler.

VI.2.4. Pad1 enzyme activity during alcoholic fermentation

A top-fermenting industrial brewing yeast strain (CMBS yeast collection) displaying high Pad1 activity and a top-fermenting yeast strain with no Pad1 activity were propagated and pitched in EBC tall tubes according to the procedure described in paragraph VI.2.3. Standard 12 °P wort (1.8 L) was pitched with 10 x 10^6 propagated yeast cells per mL in EBC tall tubes. Incubation temperature during fermentation was set at 20 °C for 12 days. For the determination of Pad1 activity, 1.5 mL of concentrated yeast suspension was centrifuged (3000 g, 3 min, 4 °C). After removal of the supernatans, the yeast pellet was washed with 1 mL physiological water. The yeast pellet was resuspended in 250 µL 50 mM phosphate buffer pH 6.0 supplemented with 0.2 mM EDTA and 250 µL glass beads (425-600 µm) were

added. Yeast cell suspensions were subjected to three Fastprep (MP Biomedicals, Illkirch, France) cycli of 15 sec with intermediate cooling on ice (2 min). Suspensions were centrifuged (15 min, 14000 rpm, 4 °C). FA was supplemented to the supernatans (200 µL) to a final concentration of 200 ppm (5 µL from a stock solution of 80 mg FA in 10 mL EtOH). After 60 min at 30 °C, the assay solution was diluted 10 times in HPLC-ECD mobile phase (pH 2) and analysed by HPLC-ECD on final 4VG concentration. Pad1 activity is expressed as the amount of 4VG (µmol) formed under the conditions of the assay per mg protein per hour. The protein concentration was determined with the Bradford assay (Bradford, 1976). Yeast extract (50 µL) was mixed with 1.45 mL Bradford reagens (Sigma-Aldrich, USA). The solution was diluted if necessary and the absorption was measured at 590 nm against a blank sample. A calibration curve using BSA (*Bovine Serum Albumine*, Sigma, USA) was constructed.

VI.2.5. Effect of temperature, pH, EtOH and FA concentration on Pad1 activity of crude cell extracts and whole cell suspensions

A top-fermenting industrial brewing yeast strain (CMBS yeast collection) with high Pad1 activity was grown in 50 mL YPD medium at 20 °C for 24 h. Yeast cells were harvested by centrifugation and Pad1 activity was assessed both on crude cell extracts (according to the procedure described in paragraph VI.2.4) and on whole cells. For the determination of Pad1 activity of whole cells, 1.5 mL of yeast suspension was centrifuged (3000 g, 3 min, 4 °C). After removal of the supernatans, the yeast pellet was washed with 1 mL physiological water. The yeast pellet was resuspended in 1.0 mL 50 mM phosphate buffer pH 6.0 supplemented with 0.2 mM EDTA and 200 ppm FA. After 60 min at 30 °C, the assay solution was diluted 20 times in HPLC-ECD mobile phase (pH 2) and analysed by HPLC-ECD on final 4VG concentration. Pad1 activity is expressed as the amount of 4VG (µmol) formed during the conditions of the assay normalised for the optical density of the yeast suspension after incubation measured at 600 nm (OD).

To assess the effect of temperature, incubation temperatures during the assay were set at 20, 30, 40, 50, 60 and 70 °C. Acetate (50 mM; pH 3.5-6.0), phosphate (50 mM; pH 6.0-8.0) and Tris assay buffers (50 mM – pH 8.0-9.0) were used for the determination of the effect of the pH on Pad1 activity between pH 3.5 and 9.0. To test the effect of ethanol on Pad1 activity, the ethanol concentration of the assay buffer was set at 0, 2, 4, 6, 8 and 10 v/v %. Substrate

inducibility was assessed by supplementing FA (0, 10, 50, 100, 500 and 1000 ppm) to the YPD medium during propagation.

VI.2.6. Effect of temperature, pH, pitching rate, C-source, FA concentration and the use of specialty malts on 4VG formation during fermentation

Unless otherwise specified, lab-scale fermentation experiments were conducted on wort according to the procedure hereby described. A top-fermenting yeast strain possessing medium Pad1(+) activity (CMBS yeast collection) was inoculated on 5 mL YPD medium and incubated at 25 °C for 24 h. After 24 h, the yeast cell suspension was transferred to 50 mL sterilised, hopped wort (12 °P). The yeast propagation was continued for 48 h at 20 °C on a rotary shaker. Yeast cells were harvested by centrifugation (3000 rpm, 4 °C, 3 min) and resuspended in physiological water. Yeast cells were pitched at 10 x 10^6 cells per mL in 350 mL wort (12 °P). Fermentations were performed at 20 °C. The fermentation process was monitored by the production of CO_2 in the water slots and sampling was performed at the end of the fermentation process after yeast flocculation. Extract content was measured to assure the finalisation of the fermentation process and samples were taken for HPLC-ECD analysis.

The effect of the fermentation temperature was assessed by setting the incubation temperature at 14, 16, 18, 20, 22 and 24 °C. Initial wort pH was varied between 4.2 and 5.7 by addition of 0.1 N HCl or NaOH. Wort was pitched with 5, 10 and 15 x 10^6 cells per mL. To assess the effect of sugar addition, wort (12 °P) was supplemented with either 3 °P glucose or maltose leading to an initial wort density of 15 °P. To investigate the effect of pure sugars on Pad1 activity, fermentations could not be conducted on wort because wort is inevitably a mixture of different kind of sugars. Fermentations were conducted on a Yeast-Peptone medium (20 g/L peptone and 10 g/L yeast extract) with 80 g/L of different kind of sugars (i.e. maltose, glucose, fructose, galactose and sucrose) supplemented with 20 ppm FA. The effect of the initial FA concentration on 4VG formation during fermentation was assessed by supplementing wort with 0, 2.5, 5, 10, 50, 250 and 1000 ppm FA. Lower concentrations (0.1, 0.2, 0.4, 0.8, 1.6, 3.2 and 6.4 ppm FA) were assessed on YPD medium (20 g/L peptone, 10 g/L yeast extract, 80 g/L glucose) because of the native FA content naturally occurring in wort. The effect of the use of specialty malts was assessed by the supplementation of extracts of three specialty malts. Extracts of Carafa (roasted malt), Caramunich (caramel malt) and Melanoidin (coloured malt) were obtained by extraction (30 min) of 18.0 g of finely milled specialty malt with 100 mL water (45 °C). Suspensions were centrifuged (15 min, 20 °C,

3000 rpm) and filtered. Specialty malt extracts (2.5 and 5 %) were added to 12 °P wort before pitching.

VI.2.7. Pilot-scale fermentation experiments: effect of applying top-pressure and effect of bottom *versus* top cropping of yeast and yeast sedimentation on 4VG formation

Two pilot-scale fermentation experiments were conducted. Each cilindroconical tank was filled with 2.5 hL wort and pitched with an industrially harvested top-fermenting *S. cerevisiae* brewing yeast strain at 5 x 10^6 cells per mL. Worts were fermented at 18 °C. During the first pilot-scale fermentation experiment, the effect of applying top-pressure on the formation of 4VG was assessed. Wort was produced according to the procedure in paragraph V.2.13. On the first fermentation tank, no pressure was applied while on the second fermentation vessel, a continuous pressure of 2 bar was applied.

To determine the effect of top *versus* bottom cropping of yeast and yeast sedimentation, wort was produced according to the procedure in paragraph V.2.13 with some minor adjustments: another batch of industrial pilsner malt was used; wort was prepared with 95 % barley malt and 5 % caramalt (50 EBC); mashing-in was at 50 °C; the pH was corrected to 5.6; after 15 min, the temperature was raised (1 °C/min) to 78 °C with a 5 min hold at 53 °C, a 40 min hold at 63 °C and a 15 min hold at 72 °C and, after filtration, the pH was adjusted to 5.2 by the addition of lactic acid. Wort (2 x 2.5 hL) was pitched in two cilindroconical tanks with a top-fermenting *S. cerevisiae* yeast strain at 5 x 10^6 cells per mL. In the first tank, yeast was top cropped each day starting from day 3 to assure that as little yeast as possible sedimented to the bottom of the tank. In the second tank, yeast was partially top-cropped (day 3) while the remaining yeast was left to settle to the cone of the tank and bottom-cropped at the end of fermentation (day 7).

VI.2.8. Headspace gas chromatography (HS-GC)

Headspace gas chromatography coupled with flame ionisation detection (GC-FID) was used for the measurement of acetate esters and ethyl esters in the fermentation products. Samples were filtered and 5 mL was collected in a 15 mL pre-cooled glass tube, which was immediately closed and cooled on ice. Samples were then analysed with a calibrated Autosystem XL gas chromatograph with a headspace-sampling unit (HS40; Perkin-Elmer, Wellesley, Mass.) and equipped with a CP-Wax 52 CB column (length, 50 m; internal diameter, 0.32 mm; layer thickness, 1.2 μm; Chrompack, Varian, Palo Alto, CA). Samples

were heated for 25 min at 60 °C in the headspace autosampler. The injection block and flame ionisation detector temperatures were kept constant at 180 and 250 °C, respectively. Helium was used as the carrier gas. The oven temperature was held at 50 °C for 5 min, then increased to 200 °C at a rate of 5 °C per min and finally held at 200 °C for 3 min. Results were analysed with Perkin-Elmer Turbochrom Navigator software.

VI.2.9. *PAD1* gene expression profile during wort fermentation with quantitative PCR

The expression level of *PAD1* was determined using qPCR. Yeast samples were collected during the first pilot-scale wort fermentation experiment from the tank to which no pressure was applied (paragraph VI.2.7.). Samples were immediately cooled on ice. mRNA extraction of pelleted cells was performed with Trizol (Invitrogen), according to the manufacturer's instructions. For each sample, 1 µg of total RNA was subject to reverse transcription (RT) using the Reverse Transcription System (Promega A3500, Madison, WI, USA). cDNA concentrations were measured and samples diluted to 100 ng/µL. The 25 µL PCR reaction was composed of 12.5 µL Power SYBR Green qPCR Master Mix (Applied Biosystems, Warrington, UK) and 1.25 µL of each primer (500 nM). 5 µL of cDNA was added to each reaction mix. The PCR program was performed on an ABI Prism 7500 Instrument: an initial incubation of 10 min at 95 °C, amplification by 40 cycles of 15 sec at 95 °C, 1 min at 60 °C. The gene for 18S rRNA (*RDN18-1*) was used as the reference gene to normalise the expression levels of *PAD1*, because the expression of this gene was found to be relatively stable during fermentation. The PCR primers were designed with the primer designing tool of SGD (www.yeastgenome.org). Primer sequences used for qPCR analysis (from 5' to 3') were: *PAD1*-qPCR-FW (ACCCAAGAGCCTTCATGACCTATT), *PAD1*-qPCR-RV (AAAAGTGTCAGCGTGGATGCCAAA), RDN18-1-qPCR-FW (CGGCTACCACATCCA AGGAA) and RDN18-1-qPCR-RV (GCTGGAATTACCGCGGCT). The expression levels were determined using ABI Prism 7500 System Gene Quantification Software (Applied Biosystems) through quantification of the SYBR Green which binds to newly synthesised double-stranded DNA. The expression levels were normalised with respect to 18S rRNA expression levels.

VI.2.10. Effect of bottle refermentation on 4VG formation

The ability of ten top-fermenting *S. cerevisiae* brewing yeast strains (CMBS yeast collection) to decarboxylate FA during wort fermentation was compared with the ability of these yeast strains to decarboxylate FA during bottle refermentation of a pilsner beer (5.2 % v/v alc., 7

EBC, 11.7 °P $E_{orig.}$, 4.9 g/L CO_2) and a blond specialty beer (7.5 % v/v alc., 13.7 EBC, 16.5 °P $E_{orig.}$, 6.6 g/L CO_2). Yeast strains were pitched on wort (350 mL, 15 °P, 20 °C) at 10 x 10^6 cells/mL. Refermentation experiments were started by the addition of 1 x 10^6 cells/mL beer and 3 g/L sucrose to beer. Bottles were incubated for 3 weeks at 23 °C. Different bottle refermentation conditions were assessed in pilsner beer by changing the amount of sugar added (0, 1, 2, 3 and 5 g/L sucrose), the yeast pitching rate (0, 0.5, 1, 2 and 3 x 10^6 cells/mL) and the pH of the beer (3.76, 3.96, 4.16, 4.36 and 4.56).

VI.2.11. Effect of repitching with top- and bottom-cropped yeast and yeast storage on 4VG formation

Yeast was cropped (top and bottom) from a pilot-scale wort fermentation experiment (paragraph VI.2.7). Top and bottom cropped yeast was repitched in wort (14.3 °P, 1.8 L) at 5 x 10^6 cells/mL in EBC tall tubes and incubated at 20 °C. Both yeast suspensions were stored at 0-4 °C for 14 days and repitched a second time in the same wort under the same conditions.

VI.2.12. Statistics

All analyses were carried out at least in duplicate (true replicates) except for the tall tube fermentation experiments with 11 top-fermenting yeast strains and the pilot-scale fermentation experiments for which duplicate samples were taken at each sampling point. Results of wort and beer analyses are expressed as the mean ± standard deviation. Differences between means were considered statistically significant when the *p*-value of the two-tailed Student's *t*-test was < 0.05 (95% confidence level) as provided by the ANOVA matrix.

VI.3. RESULTS AND DISCUSSION

VI.3.1. Screening for Pad1 phenotype in *Saccharomyces cerevisiae* and *Brettanomyces* spp. and Pad1 substrate specificity

VI.3.1.1. Semi-anaerobic screening for Pad1 phenotype among S. cerevisiae

To gain insight into the distribution of Pad1 activity of brewing yeasts, 75 top-fermenting *Saccharomyces cerevisiae* brewing yeast strains and 10 bottom-fermenting yeast strains were screened for their ability to convert FA (10 mL YPD, 100 ppm FA, 3 days, 25 °C, semi-anaerobic incubation). All bottom-fermenting brewing yeasts strains were invariably unable to convert FA. In contrast, a high incidence of Pad1 phenotype was observed among top-fermenting brewing yeast strains (figure VI.1). This corresponded with the results obtained by Perpete *et al.* (2001) who found that a high production of 4VG is a typical phenotypical property of top-fermenting yeast strains. More than 70 % of the screened top-fermenting yeasts were able to decarboxylate FA. Analogous results were obtained with wine yeasts (Shinohara *et al.*, 2000). However, a large variability in the amount of FA that can be decarboxylated was observed. Of the examined yeasts, 36 % were able to convert up to 25 % of the added FA, 25 % were able to convert between 25 and 50 % of the supplemented phenolic acid and 12 % were able to convert more than half of the FA present in the incubation medium. Only 27 % of the studied yeasts were not able to convert FA. All yeasts involved in the production of wheat beers were able to decarboxylate FA, but they were not distinctly more phenolic than other Pad1(+) top-fermenting yeasts.

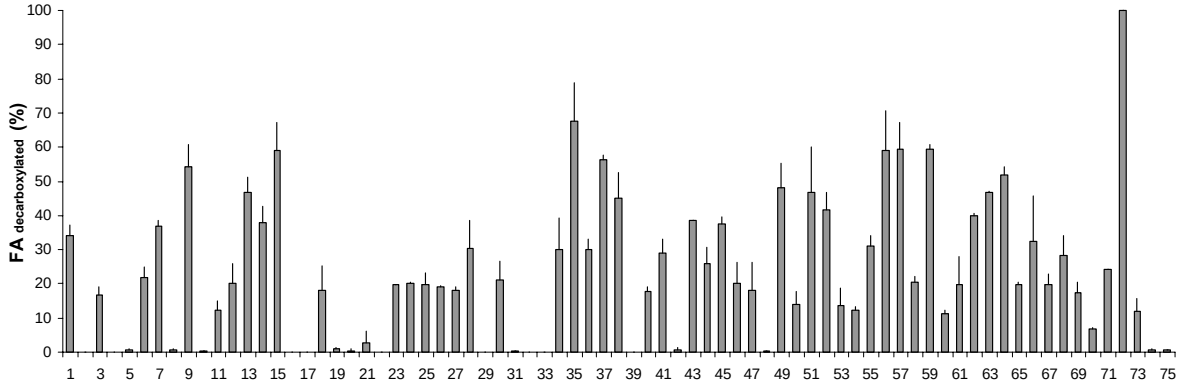


Figure VI.1. *Decarboxylation of FA (expressed % $FA_{decarboxylated}$) by 75 industrial top-fermenting* Saccharomyces cerevisiae *brewing yeast strains*

VI.3.1.2. Comparison between the semi-anaerobic screening assay and tall-tube wort fermentation experiments

The semi-anaerobic screening assay is a rapid and simple test to assess the ability of a brewing yeast strain to convert FA and can be used for testing large numbers of yeast on a short time interval. However, it is important to know whether the test is representative for the Pad1 activity of brewing yeast strains during wort fermentation. Possibly, some yeasts showed activity only under semi-aerobic growth or others did not show activity in the screening assay but are able to decarboxylate FA during alcoholic fermentation. To assess the accurateness of the screening assay, decarboxylase activity of 11 top-fermenting yeast strains, exhibiting different Pad1-activities during the semi-anaerobic screening procedure, was assessed during fermentation of 12 °P wort supplemented with 3 °P glucose and 20 ppm (0.1 mM) FA in EBC tall tubes. Also during these fermentation experiments, a large variability in Pad1-activity between different yeast strains was observed (figure VI.2). While some yeast strains were unable to convert FA during alcoholic fermentation, others where able to convert all of the added FA within 4 days (corresponding to 15.5 ppm 4VG).

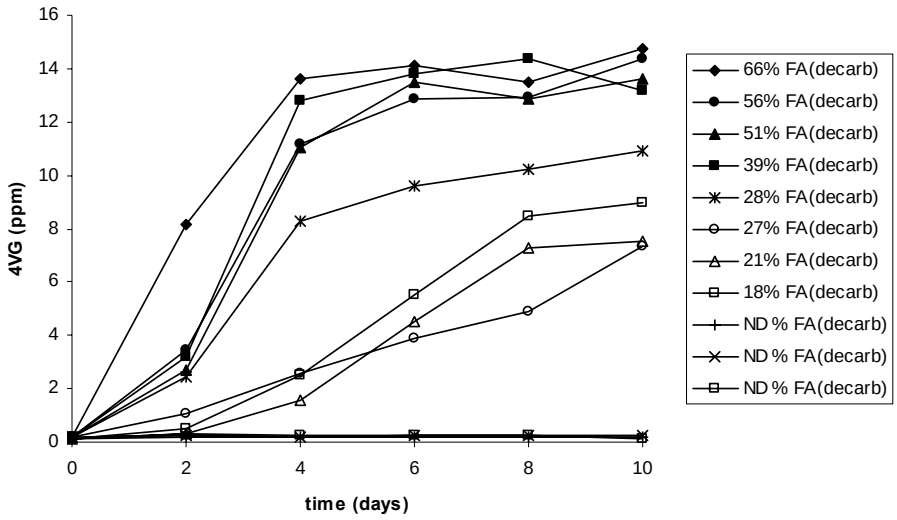


Figure VI.2. *Evolution of 4VG (ppm) during wort fermentation by 11 top-fermenting yeast strains. The legend expresses the amount of FA decarboxylated during the semi-anaerobic screening procedure as (%FA(decarb)) for Pad1-phenotype on YPD (ND: not detectable)*

A good correlation was found between the amount of FA decarboxylated during the semi-anaerobic screening assay and the amount FA decarboxylated during alcoholic fermentation of wort (figure VI.3). When the final 4VG concentration during fermentation (after 10 days) was plotted against the % FA decarboxylated during the screening, a good correlation (R^2

0.85) was found. However, since more than one yeast strain did convert the total amount of FA supplemented, it was not possible to discriminate all decarboxylase activities during fermentation with one measurement of the 4VG concentration in time. When the initial speed of 4VG conversion during fermentation (4VG concentration after 2 days) was taken into account, a R^2 of 0.74 was found. Finally, to take both the initial speed of FA conversion and the final amount of 4VG into account, the sum of the 4VG concentration in wort after 2 days and after 10 days was taken as a measure of Pad1 activity during fermentation. When this value was compared to the results of the screening assay (% FA decarboxylated) a strong correlation was obtained ($R^2 = 0.94$). Hence, the semi-anaerobic screening assay can be used as a first step in the selection of a brewing yeast strain concerning its Pad1 activity.

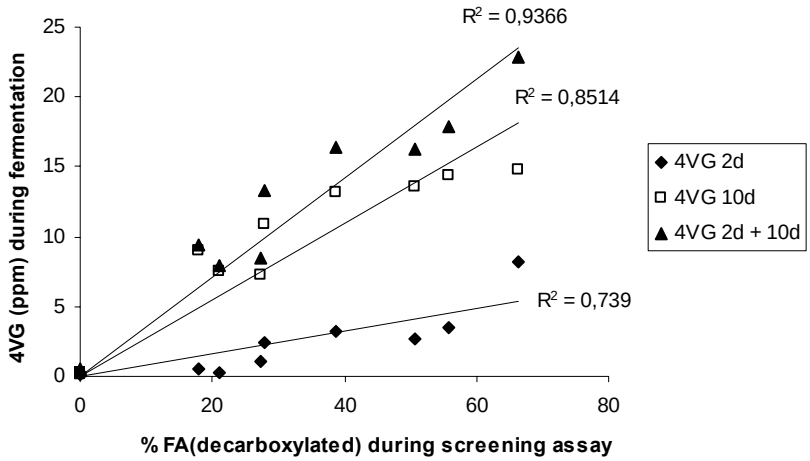


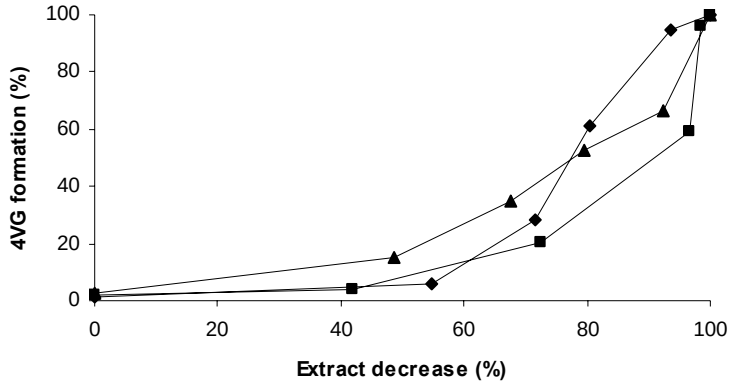
Figure VI.3. *Comparison between the Pad1 activity of 11 top-fermenting brewing yeast strains during the semi-anaerobic screening procedure (expressed as % FA(decarboxylated) and during wort fermentation (expressed as amount of 4VG formed during the wort fermentation process after 2 and/or 10 days)*

Since the added FA far exceeds the level of free FA in wort, the enzymatic decarboxylation of phenolic acids during fermentation can account for the high levels of volatile phenols often encountered in top-fermented wheat beers, blond specialty beers and dark specialty beers. In contradiction to what was suggested by Coghe *et al.* (2004a), no yeast strains possessing cinnamoyl esterase activity during fermentation were found (results not shown). The conversion of FA to 4VG was found to proceed approximately stoichiometrically. No formation of dehydroferulic acid was found as reported by some authors (Huang *et al.*, 1993).

VI.3.1.3. Time course of 4VG formation and FA toxicity

When the 4VG formation (expressed as a percentage) was plotted against the evolution of the extract content (% extract decrease) for the three Pad1(+) yeasts, which were not subject to

substrate limitation during wort fermentation, it can be seen that the majority of 4VG was formed during the second half of the fermentation process (figure VI.4). More than 80 % of the final 4VG content in wort was formed after more than 50 % of the fermentable extract was converted.



Figuur VI.4. *4VG formation during wort fermentation related to the extract decrease for three yeast strains not subject to a limitation in FA availability during the fermentation process*

Yeast tolerance against FA was examined by incubating the 11 selected top-fermenting yeast strains with increasing concentrations of FA (10, 20, 50, 100 and 1000 ppm) according to the semi-anaerobic screening procedure. After three days of incubation, the dry weight of the obtained yeast suspension was determined and compared to a blank sample which did not contain FA. Only at the highest concentration (1 g/L), inhibition of the yeast growth was found. Since this concentration far exceeded levels encountered in wort (< 10 ppm), this inhibitory effect will not have any consequence on yeast performance during wort fermentation and is of little practical relevance in the brewery industry. Moreover, the growth inhibition was inversely correlated with Pad1 activity of the examined yeast strains, indicating that the decarboxylase activity may indeed provide protection against HCA's toxicity.

VI.3.1.4. Screening of FA decarboxylase and 4VG reductase activity among Brettanomyces/Dekkera *spp.*

To confirm that the ethylphenols found in beers with mixed or spontaneous fermentation (chapter III) could be attributed to the presence of *Brettanomyces/Dekkera* spp., 11 yeast strains purified from lambic beers (Kumara *et al.*, 1991; Martens *et al.*, 1997) belonging to the species *bruxellensis*, *custersii*, *anomalus* and *lambicus* were screened for their ability to

convert FA (10 mL YPD, 100 ppm FA, 3 days, 25 °C). All strains were able to convert FA (conversion between 60.4 and 100 %). After incubation, 4EG was the most dominant volatile phenol present (47.3-78.4 ppm) whereas 4VG was only present in small amounts (not detectable levels up to 4.4 ppm). No differences between the different subspecies were found. The decarboxylase activity of the *Brettanomyces/Dekkera* yeast strains was significantly higher than that of the studied *Saccharomyces cerevisiae* yeast strains. Notwithstanding, since in most experimental set-ups FA can still be detected while 4VG cannot, the decarboxylation seems to be the rate-limiting step in the formation of the ethylphenols.

VI.3.1.5. In vivo *substrate specificity of Pad1*

The *in vivo* substrate specificity of 11 top-fermenting yeast strains was assessed according to the semi-anaerobic screening procedure on YPD supplemented with 1 mM pCA, FA and SA. Results are represented in table VI.1.

Table VI.1. *Percentage of pCA, FA and SA decarboxylated by 11 top-fermenting yeasts during semi-anaerobic screening*

	% decarboxylated		
	pCA	**FA**	**SA**
Yeast 1	24	51	ND
Yeast 2	12	18	ND
Yeast 3	33	56	ND
Yeast 4	ND	ND	ND
Yeast 5	10	21	ND
Yeast 6	24	27	ND
Yeast 7	ND	ND	ND
Yeast 8	ND	ND	ND
Yeast 9	24	28	ND
Yeast 10	39	66	ND
Yeast 11	29	39	ND

None of the yeasts was able to decarboxylate SA. This explains why no 4VS was detected in the analysed beer samples. Probably, 4VS is present but arises only in small concentrations from the thermal decarboxylation of SA. The yeasts that possessed decarboxylase activity *versus* FA did so *versus* pCA but to a lesser extent. Similar results were obtained in EBC tall tube fermentation experiments with three top-fermenting yeast strains with FA, pCA and SA added to fermenting wort (results not shown). The substrate specificity of the Pad1 enzyme is reflected in the conversion of pCA, FA and SA in commercial beers (chapter III).

VI.3.2. Pad1 activity during wort fermentation

A top-fermenting yeast strain displaying high Pad1 activity based on the results of the screening and the tall tube fermentation experiments was pitched at 10 x 10^6 cells per mL on standard 12 °P wort (1.8 L). Incubation temperature during fermentation was set at 20 °C for 12 days. A fermentation with a Pad1(-) yeast strain was conducted in parallel as a control sample. Samples were taken initially twice a day (day 1-4) and then every day (day 5-12) to assess Pad1 activity. The course of the fermentation was followed by daily measurements of the apparent extract content (°P). The fermentation with the Pad1(-) control yeast proceeded normally reaching final attenuation within one week. 4VG formation could not be detected during fermentation and no Pad1 activity was detected in crude cell extracts from yeast cells sampled during fermentation. Concerning the Pad1(+) yeast strain, the evolution of extract content (°P) and the Pad1 activity (µmol 4VG/mg protein) during fermentation is displayed in figure VI.5. Initially the wort contained 2.2 ppm FA. This was converted completely to 4VG at day 3 and day 4. Due to this substrate depletion, wort 4VG concentration could not be used as a measure of Pad1 activity during fermentation.

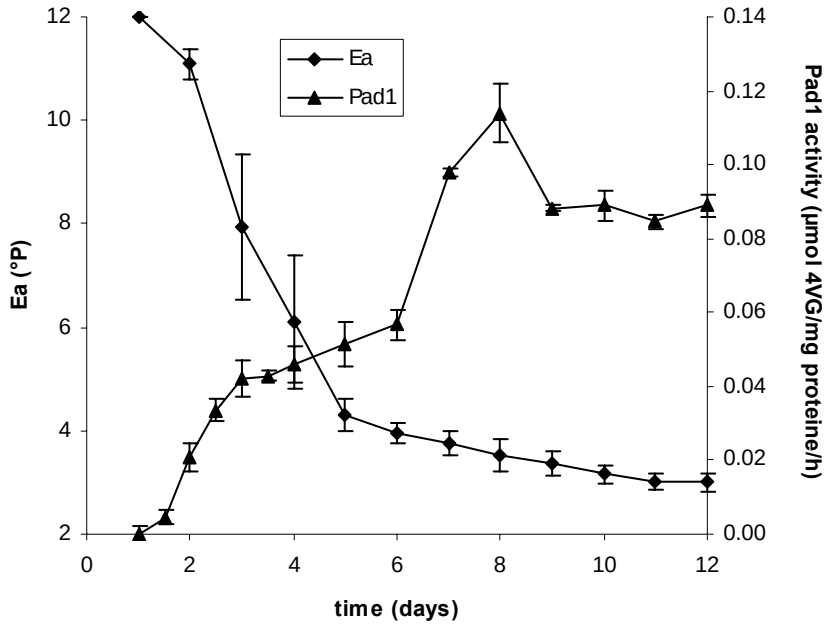


Figure VI.5. *Evolution of apparent extract content (°P) and Pad1-activity (µmol 4VG per mg protein per h) during wort fermentation with a Pad1(+) top-fermenting industrial yeast strain*

At the start of the fermentation process, no Pad1-activity could be detected. During fermentation, the activity first increased reaching maximum activity at day 8. Clearly, yeast cells displayed the highest Pad1 activity after the majority of the fermentable extract was

consumed. This is consistent with the results presented in paragraph VI.3.1.3 were it was seen that, when the FA availability is not limiting, the majority of 4VG is formed during the second half of the fermentation process when most of the fermentable extract was converted. When all fermentable sugars were converted, Pad1 activity decreased slightly and maintained a constant value until the end of the monitoring. The experimental set-up has been repeated for another Pad1(+) top-fermenting yeast strain. Analogous results were obtained. The maximum Pad1 activity reached during fermentation was consistent with the results of the screening assay. While the first yeast strain displayed a maximum Pad1 activity of 0.11 µmol 4VG/mg protein (figure VI.5) and converted 66 % FA during the screening procedure; the second yeast strain reached a maximum Pad1 activity of 0.03 µmol 4VG/mg protein while converting 28 % FA during the screening procedure.

VI.3.3. Effect of temperature, pH, EtOH and FA concentration on Pad1 activity of crude cell extracts and whole cell suspensions

The temperature and pH dependence, ethanol tolerance and substrate induction of the Pad1 activity of both yeast crude cell extracts (left) and suspensions with intact yeast cells (right) are depicted in figure VI.6. Concerning the temperature, no significant difference was found between the Pad1 activity at 20 and 30 °C. Probably the optimal temperature for the decarboxylase activity is situated within this temperature range. When the temperature was further increased, Pad1 activity of the crude cell extracts and the whole cell suspensions decreased steadily. Concerning the pH dependence, maximal Pad1 activity was obtained at pH 6.0 for the crude cell extracts. The Pad1 activity of the whole cell suspensions showed a somewhat broader optimal pH range with maximal activities at pH 5.5 and 5.0. This is due to the ability of yeast cells to maintain an intracellular pH homeostasis over a quite broad range of external pH's (Valli *et al.*, 2005). At pH 3.5 and 9.0, no Pad1 activity could be detected in the crude cell extracts while the whole cell suspension still displayed a quite high Pad1 activity at pH 3.5. Within the range of wort pH during fermentation, Pad1 activity of cell suspensions remained quite high. The ethanol concentration had a negative effect on Pad1 activity. Activity decreased with increasing ethanol concentrations, both in crude cell extracts and suspensions of intact yeast cells. Concerning the influence of the FA concentration during yeast propagation, a clear inductive effect could be seen. When yeast cells were grown on YPD medium without FA, crude cell extracts as well as whole yeast cells displayed Pad1 activity. However, increasing the FA concentration during the propagation phase, clearly increased the Pad1 activity of both the crude yeast cell extracts and the cell suspensions with

intact yeast cells. The mechanism by which this increase is triggered and whether it is correlated with *PAD1* expression is unknown, but clearly the presence of FA induced Pad1 activity probably to counteract its toxicity.

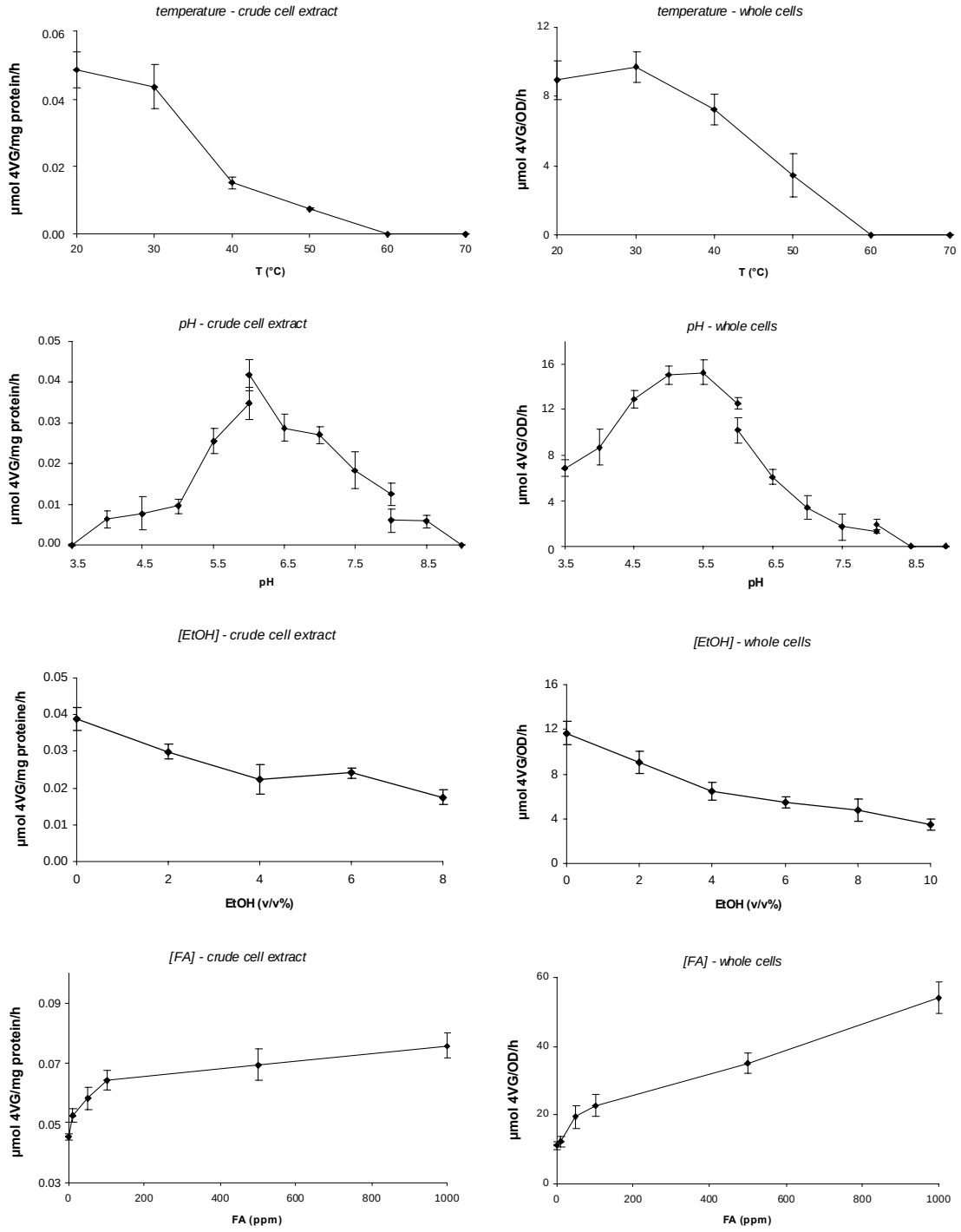


Figure VI.6. *Effect of temperature, pH, EtOH and FA concentration on Pad1 activity of yeast crude cell extracts (µmol 4VG/mg protein/h- left) and suspensions of intact yeast cells (µmol 4VG/OD/h - right)*

VI.3.4. Effect of temperature, pH, pitching rate, C-source, FA concentration and the use of specialty malts on 4VG formation during laboratory-scale fermentations

For the laboratory-scale fermentation experiments, a yeast strain with medium Pad1(+) activity, which was able to convert about half of the FA present in wort under standard laboratory-scale wort fermentation experiments (wort 12 °P, pitching 10 x 10^6 per mL, 20 °C) was selected. Both the final 4VP and 4VG concentration in wort were determined by HPLC-ECD analysis. Because of the highly parallel evolution of both volatile phenols, only the results of the 4VG formation, being the dominant volatile phenol in beer, is reported.

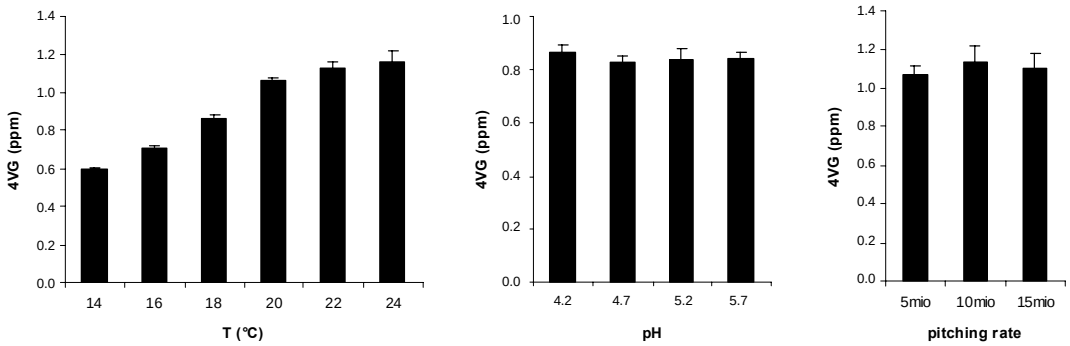


Figure VI.7. *Effect of the temperature, wort pH and yeast pitching rate on the formation of 4VG during wort fermentation*

With increasing fermentation temperatures, an increase in the 4VG concentration in wort was observed. At 24 °C, the final 4VG concentration was almost twice that found at 14 °C. The elevated concentrations found at higher temperatures can be due to both an increased Pad1 activity (for which an optimal activity between 20 and 30 °C was observed) or due to the more rapid yeast cell growth at higher temperatures. Within the examined pH range (4.2-5.7), no effect was found on the final 4VG concentration in wort. This was probably due to the ability of yeast cells to maintain its intracellullar pH fairly constant over a broad pH range (Valli *et al.*, 2005) as was observed with the Pad1 activity of whole cell suspensions. The initial yeast cell pitching rate (5, 10 or 15 x 10^6 cells/mL) did not affect the formation of 4VG during fermentation. Since yeast cell counts can raise more than tenfold during the initial stages of the fermentation process, the initial differences were probably smoothed out by the end of the fermentation process (when the lion's share of the 4VG formation took place).

The effect of the initial FA concentration on 4VG formation during fermentation was assessed by supplementing wort with 0, 2.5, 5, 10, 50, 250 and 1000 ppm FA. Lower concentrations (0.1, 0.2, 0.4, 0.8, 1.6, 3.2 and 6.4 ppm FA) were assessed on synthetic YPD medium because of the native FA content naturally occurring in wort (figure VI.8). Clearly,

elevated initial FA concentrations led to increased 4VG concentrations both in YPD and in wort. The increased decarboxylation of FA can be due to both the increased substrate availability and due to the substrate induction effects already described. At the levels relevant in the brewery industry (≤ 10 ppm), the extent of the decarboxylation of FA was highly correlated with its initial concentration ($R^2 > 0.99$) both in wort and in YPD medium. At higher FA concentrations in wort, the decarboxylation rate decreased rapidly. The results of these experiments are highly relevant, since they validate the possibility of optimising final volatile phenol content in beer by controlling the release of HCA's in during brewhouse operations.

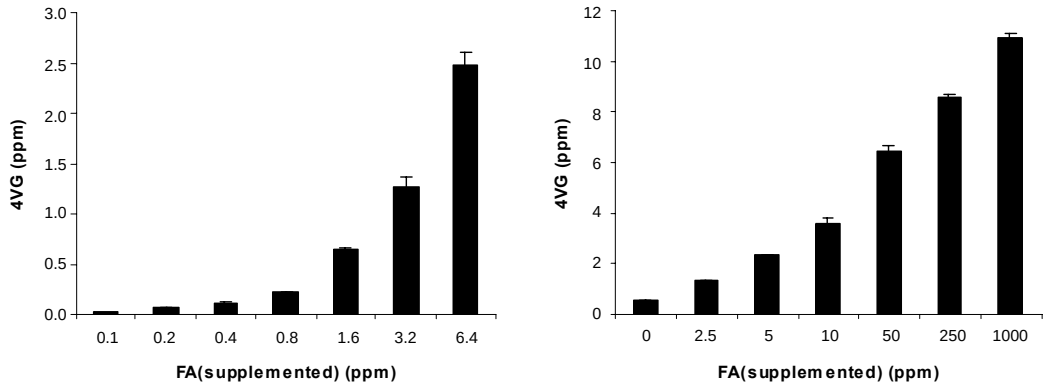


Figure VI.8. *Effect of the FA concentration on the formation of 4VG during fermentation of synthetic YPD-medium (left – low concentrations) and of wort (right – high concentrations)*

The effect of the use of specialty malts was assessed by the supplementation of extracts of three specialty malts during wort fermentation (figure VI.9). Extracts of Carafa (roasted malt), Caramunich (caramel malt) and Melanoidin (coloured malt) (2.5 and 5 %) were added to 12 °P wort before pitching. All worts had equal initial concentrations of FA (1.83 ± 0.02 ppm) apart from the wort supplemented with 5 % Carafa extract, which contained a slighlty lower concentration (1.79 ppm). The worts supplemented with the roasted Carafa malt extract did also contain a slightly elevated 4VG content (0.17 and 0.22 ppm for the 2.5 and 5 % supplementation respectively compared to 0.13 ppm for the other worts). Clearly, the worts that were supplemented with the roasted malt extract, exhibited lower levels of 4VG at the end of the fermentation process. In the wort supplemented with 5 % Carafa malt extract, the 4VG content was only half that of the blank wort sample. The increase in 4VG concentration corresponded approximately with the decrease in FA. Hence, the observed difference cannot be attributed to the possible bonding of FA to roasted malt extract compounds by which it may have had become unavailable for decarboxylation. It seems that the Pad1 enzyme itself

was affected by the roasted malt extract. The observed decrease in the degree of decarboxylation in worts supplemented with specialty malt extracts can account for the observed discrepancy in 4VG content between blond and dark specialty beers.

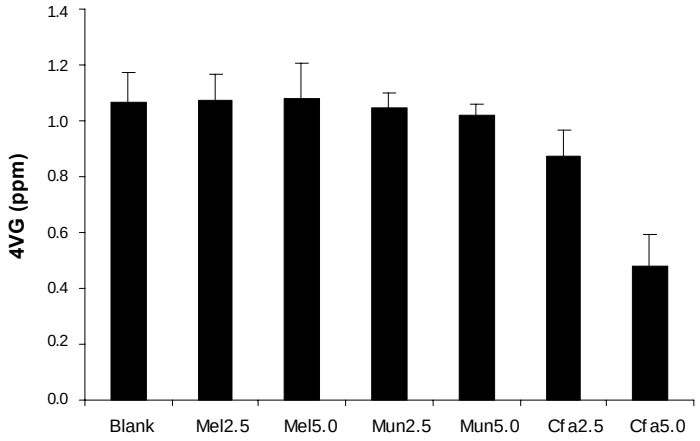


Figure VI.9. *Effect of the addition of specialty malt extracts (2.5 and 5 %) on the formation of 4VG during wort fermentation (Mel: Melanoidin – Mun: Caramunich – Cfa: Carafa)*

To assess the effect of sugar addition, wort (12 °P) was supplemented either with 3 °P glucose or maltose leading to an initial wort density of 15 °P. To investigate the effect of pure sugars on Pad1 activity, fermentations could not be conducted on wort because wort is inevitably a mixture of different kind of sugars. Fermentations were conducted on a synthetic YP medium supplemented with maltose, glucose, fructose, galactose or sucrose and with 20 ppm FA.

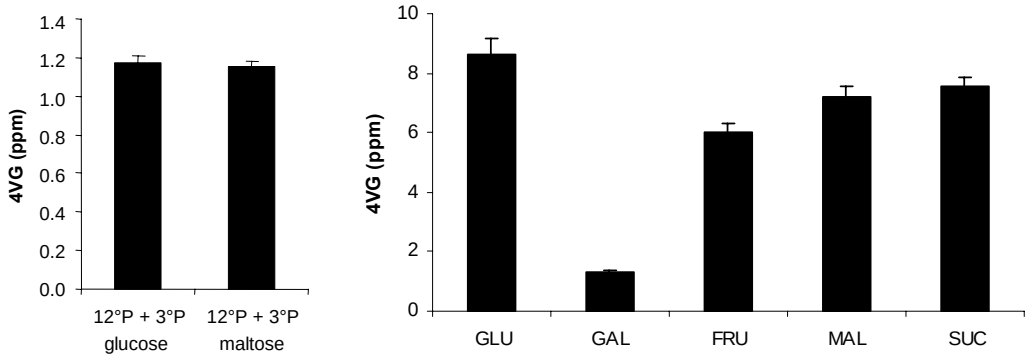


Figure VI.10. *Effect of the carbon source: supplementation of 3 °P glucose and maltose during wort fermentation (left) and fermentations of synthetic YP-medium with 5 different sugars (right) (GLU: glucose – GAL: galactose – FRU: fructose – MAL: maltose – SUC: sucrose)*

The addition of 3 °P glucose or maltose to wort (12 °P) did not affect the final 4VG concentration. Since 4VG is formed mainly at the end of fermentation, the initial altering of

the sugar wort profile would probably have had little effect given the sequential sugar uptake by yeast. By the start of FA decarboxylation, the wort sugar profile was approximately the same for both worts. However, when fermentations were conducted on synthetic YP medium with different sugars, differences in final 4VG concentration were found. The highest degree of FA decarboxylation was found on the glucose medium. Concerning the other monosaccharides, fructose and especially galactose, a significant lower amount of FA was decarboxylated. On maltose, the major wort sugar, and sucrose, a disaccharide of fructose and glucose, which is readily converted to the constituent monosaccharides, the Pad1 enzyme displayed intermediate activity. How these sugars intervene in the regulation of the Pad1 enzyme activity is not known. However it is clear that these results cannot explain why the Pad1 enzyme displayed optimal activity near the end of fermentation.

VI.3.5. Pilot-scale fermentation experiments

VI.3.5.1. Effect of applying top-pressure on 4VG formation during wort fermentation in cilindroconical tanks

Two cilindroconical tanks were filled with 2.5 hL wort produced according to the procedure described in paragraph V.2.13. and pitched with an industrially harvested top-fermenting *S. cerevisiae* yeast strain. The evolution of the extract content and the 4VG formation during fermentation (top) and the time-course of the 4VG formation *versus* the extract decrease (bottom) are represented in figure VI.11. Frequently, top pressure is applied to cilindroconical tanks preventing overfoaming of the tanks during fermentation and allowing a higher filling volume of the tanks. On the first CCT, no counter pressure was applied (0 bar) while on the second CCT a counter pressure of 2 bar was applied. The pressure was led to increase gradually by the production of carbon dioxide by the fermenting yeast strain reaching a final pressure of 2 bar within 1 day. Wort initially contained 2.3 ppm FA and 0.14 ppm 4VG. Both fermentations proceeded normally reaching final attenuation within 5 days. The fermentation rate was only slightly delayed in the fermentation tank to which top pressure was applied. The evolution of the yeast cell counts in both tanks was identical. However, the final 4VG concentration in the tank to which top-pressure was applied was 43 % lower than the 4VG concentration in the tank to which no pressure was applied. Rather than a direct inhibitory effect due to the pressure applied, the decrease in 4VG formation was probably due to a secondary effect, namely an increase in carbon dioxide solubility at higher pressures. At atmospheric pressure and 18 °C, the solubility of carbon dioxide is ± 2 g/L, while at 2 bar the solubility equals 5.2 g/L. Possibly enzyme product inhibitory effects come

into play retarding the formation of volatile phenols (which would lead to the release of additional CO_2) when pressure is applied. When the 4VG formation (expressed as a percentage of the total amount of 4VG formed during fermentation) was plotted against the extract decrease, it was again observed that 4VG is mainly formed at the end of the fermentation process when all fermentable sugars are consumed. This suggests that the Pad1 enzyme might be involved in the course of events triggered upon the stress presented by nutrient depletion. After 7 days, beers were cooled (2 °C) for one week. During conditioning, the additional increase of 4VG was low (3 % and 4 % of final 4VG concentration for the tank with and without top-pressure, respectively).

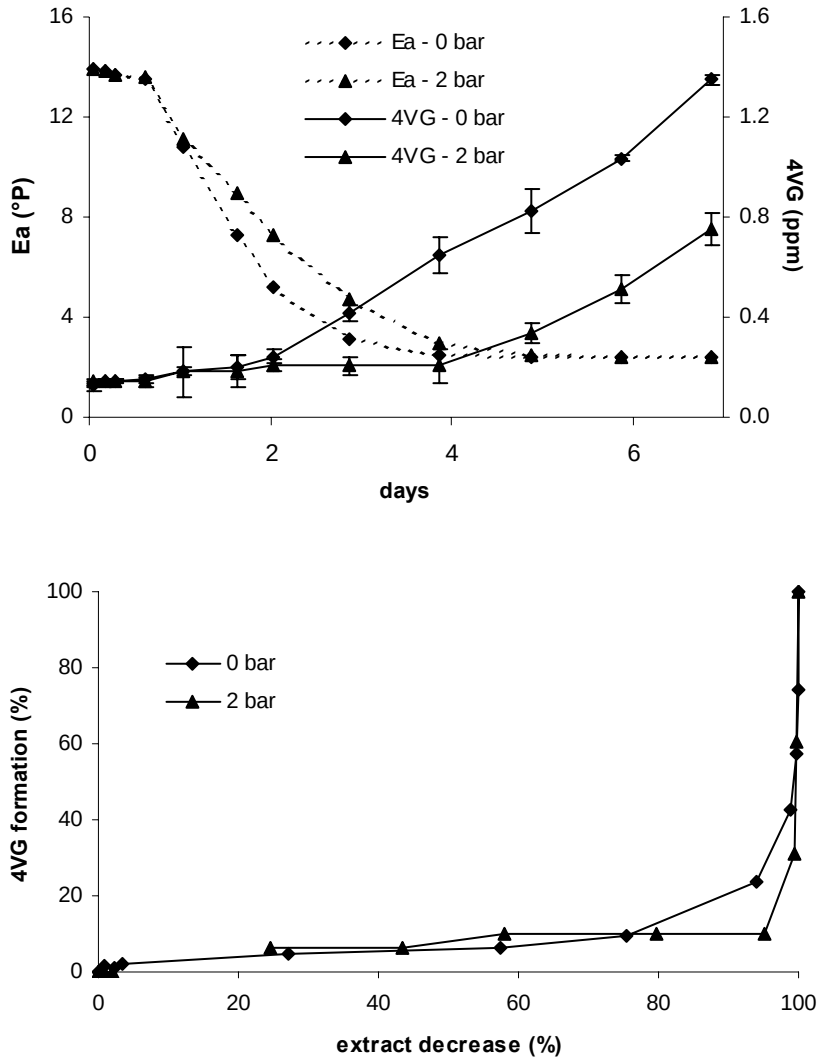


Figure VI.11. *Effect of applying top-pressure (2 bar) on 4VG formation during wort fermentation in cilindroconical tanks: evolution of extract content and 4VG formation during fermentation (top) and time-course of 4VG formation* versus *extract decrease (bottom)*

Concerning the results presented, the following question arises: “Is applying top pressure an effective means of controlling the 4VG aroma appreciation in beer?” Sensory tests of both beers were carried out using a trained panel of 11 assessors. Beers were presented at random in one session to the panellists. Different aspects of aroma and taste were evaluated by giving a score from 0 to 8. A score of 0 meant the particular flavour was absent while a score of 8 meant the particular flavour was extremely strong. Both beers were also subjected to headspace GC analysis to evaluate other important beer flavour constituents. The results of the sensory tests are shown in figure VI.12. The results of the analytical tests are presented in table VI.2.

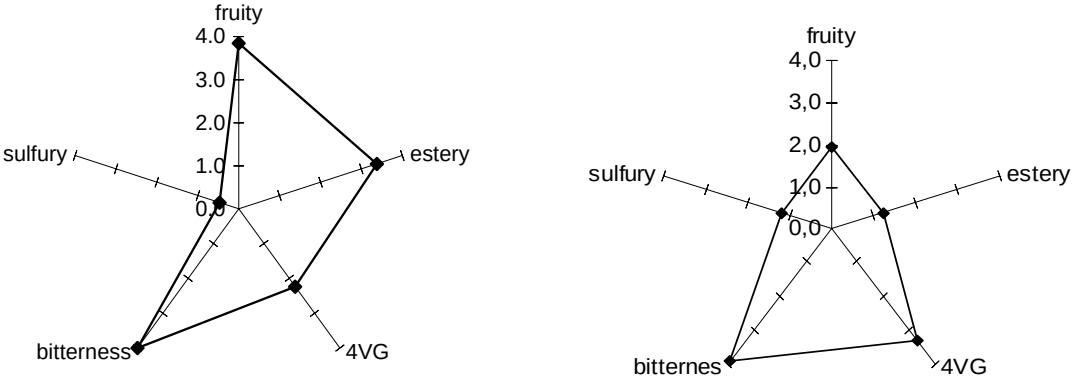


Figure VI.12. *Effect of applying top-pressure during wort fermentation in cilindroconical tanks on the sensory profile of a blond specialty beer without applying pressure (left) and with applying pressure (right)*

Table VI.2. *Effect of applying top-pressure during wort fermentation in cilindroconical tanks on 4VG and ester concentrations of a blond specialty beer*

Flavour compound	Without pressure (ppm)	With pressure (ppm)	Decrease by applying pressure (%)
4VG	1.39	0.78	43
Ethyl acetate	38.3	14.0	63
Isoamyl acetate	4.17	0.81	81
Ethyl caproate	0.18	0.08	56
Ethyl caprylate	0.41	0.20	51
Ethyl caprate	0.06	0.04	33

Although the beer to which no pressure had been applied, contained the highest level of 4VG (1.4 ppm), it received a lower quotation on phenolic aroma intensity during the sensory test than the beer which was produced with counter-pressure that only contained 0.8 ppm 4VG. On the contrary, estery and fruity flavour intensities were scored higher for the first beer than for the second beer. Headspace GC results showed that not only volatile phenolic flavour

compounds decreased when pressure was applied, but also flavour active ethyl and acetate esters which impart a fruity and estery flavour to beer, decreased significantly. Dissolved carbon dioxide is known to reduce the formation of both higher alcohols and esters (Landaud *et al.*, 2001; Shen *et al.*, 2003) Two important flavour-active esters decreased to a concentration below their flavour threshold in beer: ethyl acetate (30-33 ppm – fruity solvent-like) and isoamyl acetate (1.2-1.6 ppm – banana, pear). Because esters significantly decreased, the phenolic flavour impression, despite the decrease in 4VG content, became more dominant in the beer sensory profile. The first beer ranked higher in final score and was described as being more balanced. It is clearly important to balance the phenolic flavour with an appropriate amount of fruity and estery flavours. Both beers were also presented to a consumer panel (76 members). The vast majority (86 %) picked the beer produced without counter-pressure as their favourite beer. In practice, not only the applied top-pressure needs to be taken into account. In large cilindroconical fermentation vessels, a hydrostatic pressure of 1 bar does apply with every 10 m of fluid height. This results in an additional increase in the concentration of dissolved carbon dioxide.

VI.3.5.2. Effect of bottom versus *top cropping of yeast and yeast sedimentation*

The conversion of horizontal fermentation tanks to cilindroconical tanks has altered the way of yeast harvesting significantly. While in horizontal tanks top-fermenting yeast strains flocculate and aggregate at the surface of the fermentation tank, cilindroconical tanks open up better possibilities for yeast harvesting and recuperation by letting the yeast cells, after the initial flotation phase, sediment to the cone of the cilindroconical tank. Since the 4VG formation occurs during the final phase of the fermentation process, it was hypothesised that the yeast sedimentation step might be important in determining final volatile phenol levels in beer.

To determine the effect of top *versus* bottom cropping of yeast and yeast sedimentation, wort (2 x 2.5 hL) was pitched in two cilindroconical tanks with a top-fermenting *S. cerevisiae* yeast strain at 5 x 10^6 cells per mL. In the first tank, yeast was top-cropped (starting from day 3) each day to assure that as little yeast as possible sedimented to the bottom of the tank. In the second tank, yeast was partially top-cropped (day 3) while the remaining yeast was left to settle to the cone of the tank and bottom-cropped at the end of fermentation (day 7). The initial wort contained 1.63 ppm FA and 0.19 ppm 4VG. The two fermentations proceeded equally good, reaching final attenuation at day 5 (figure VI.13).

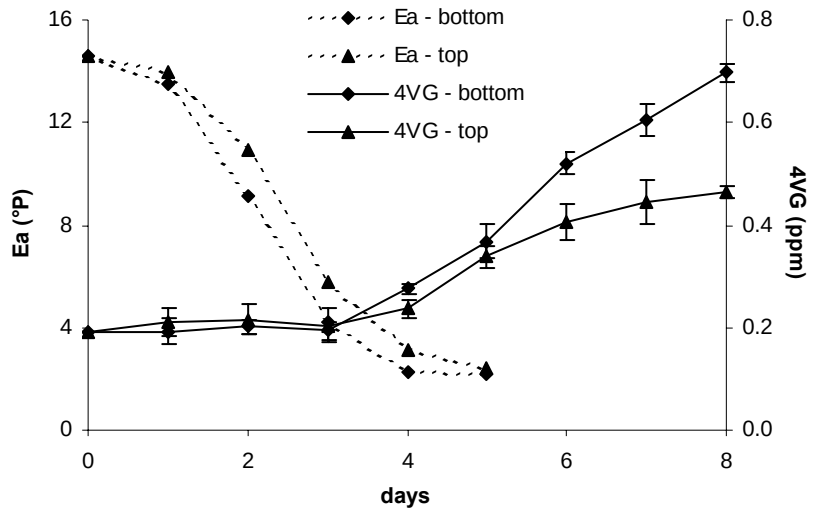


Figure VI.13. *Effect of top-cropping* versus *bottom-cropping and yeast sedimentation on the formation of 4VG during wort fermentation in cilindroconical tanks: evolution of apparent extract content and 4VG formation during fermentation*

Until day 5, the 4VG concentrations were not significantly different. On reaching final attenuation, CO_2 formation ceased and yeast cells started to sediment to the cone of the cilindroconical tank. While the yeast in one tank was regularly top-cropped preventing yeast sedimentation, the yeast on the other tank was led to sediment. More than half of the 4VG formed by enzymatic decarboxylation of FA was formed during this sedimentation phase. When top-cropping was performed regularly, the final 4VG concentration was 50 % lower than when bottom-cropping was applied. Hence, partly top-cropping the yeast near the end of fermentation can significantly decrease the final 4VG concentration. However, care has to be taken to leave enough yeast into the tank to assure a good final attenuation and a sufficient reduction in diacetyl levels.

VI.3.6. *PAD1* gene expression profile during fermentation

The expression level of *PAD1* during fermentation was determined using qPCR. Yeast samples were collected during the first pilot-scale wort fermentation experiment from the tank to which no pressure was applied (paragraph VI.2.7.). The expression levels were normalised with respect to *18S rRNA* expression levels (figure VI.14). The gene expression of *PAD1* was at its highest at the beginning of the fermentation. During fermentation, it steadily decreased. *PAD1* gene expression could clearly not be correlated to the time course of 4VG formation or to the evolution of the Pad1 enzyme activity during fermentation (figure VI.11). This confirmed the results of Clausen *et al.* (1994) and Smit *et al.* (2003) who showed that overexpression of *PAD1* could not be correlated to an elevated enzyme activity and that

post-transcriptional regulation is critical for the functioning of Pad1. Application of the semi-anaerobic screening procedure (paragraph VI.2.2) to investigate the ability to decarboxylate FA by the wild type of the laboratory yeast strain BY4742 (*MAT***a** *his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*) and its *PAD1* deletion mutant (*MAT***a** *his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 pad1Δ0::KAN*^{r}) of the Resgen/Invitrogen Belgium yeast collection, showed that deleting the *PAD1* gene completely eliminated the yeast strain's ability to convert FA (results not shown). This excludes the possibility that the discrepancy between the *PAD1* gene expression and the Pad1 enzyme activity is due to the presence of *PAD1* homologues in the yeast genome.

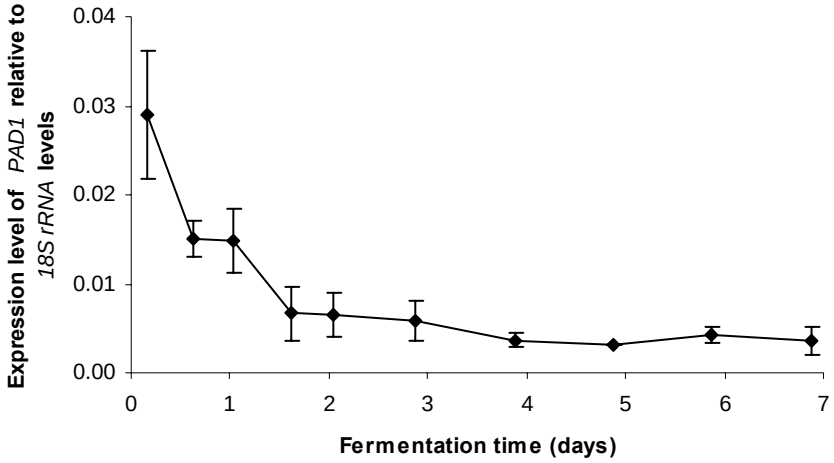


Figure VI.14. *Gene expression profile of* PAD1 *during fermentation as determined by qPCR (expression levels are normalised with respect to* 18S rRNA *levels)*

VI.3.7. Effect of bottle refermentation on 4VG formation

The ability of ten top-fermenting *S. cerevisiae* yeast strains to decarboxylate FA during wort fermentation was compared with the ability of these yeast strains to decarboxylate FA during bottle refermentation of a pilsner beer (5.2 % v/v alc., 7 EBC, 11.7 °P $E_{orig.}$, 4.9 g/L CO_2) and a blond specialty beer (7.5 % v/v alc., 13.7 EBC, 16.5 °P $E_{orig.}$, 6.6 g/L CO_2). The results of the final 4VG concentration are shown in figure VI.15. The wort, pilsner beer and blond specialty beer did initially contain 3.1 ppm, 3.0 ppm and 1.9 ppm FA, respectively. Three yeast strains did not decarboxylate FA and can be considered to be Pad1(-). The other seven yeast strains were able to decarboxylate FA during the wort fermentation experiment and can be classified as Pad1(+). For each yeast strain, the formation of 4VG during the pilsner beer refermentation was lower than during the wort fermentation experiment although both media had an equal FA content. Refermentation of the blond specialty beer hardly increased 4VG levels by any of the examined yeast strains. Hence, formation of 4VG during bottle refermentation was less pronounced than during the primary wort fermentation. The

discrepancy between both can be used to optimise final volatile levels in beer. The observed differences between the wort fermentation and the refermentation of both beers can partly be explained by the use of substantially lower yeast counts during bottle refermentation as compared to wort fermentation and the less pronounced yeast cell multiplication during bottle refermentation. However, the degree of FA decarboxylation during refermentation was also highly dependent on the beer medium. Differences observed between both beers can be explained by the different carbon dioxide content of both beers. While in the pilsner beer, an initial carbon dioxide content of 4.9 g/L corresponded to a pressure of 2.3 bar at 23 °C, the blond specialty beer initially contained 6.6 g/L corresponding to 3.3 bar at 23 °C. The differences in 4VG formation between both beers can probably be attributed to the same mechanisms that inhibited the formation of 4VG during primary wort fermentation with application of counter-pressure. Hence, bottle refermentation alone may not always be the most appropriate means of achieving a perceivable phenolic flavour in beer. The specific beer medium and the yeast strain need to be carefully selected.

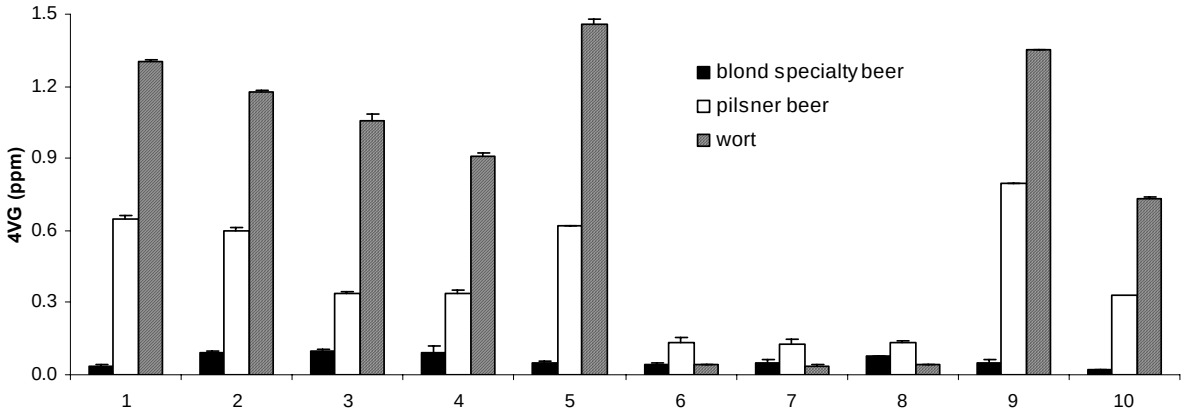


Figure VI.15. *4VG formation with 10 top-fermenting yeast strains during wort fermentation and bottle refermentation of a pilsner beer and a blond specialty beer*

Different bottle refermentation conditions during bottle conditioning of a pilsner beer were assessed by changing the amount of sugar added (0, 1, 2, 3 and 5 g/L sucrose), the yeast pitching rate (0, 0.5, 1, 2 and 3 x 10^6 cells/mL) and the pH of the beer (3.76, 3.96, 4.16, 4.36 and 4.56). The results are depicted in figure VI.16. The concentrations are normalised to the "standard" bottle refermentation condition (pH 4.16, 3 g/L sucrose and 1 x 10^6 cells/mL). In contrast to the primary wort fermentation, the yeast pitching rate was found to affect the final 4VG concentration in beer significantly. During primary fermentation, yeast cell counts may rise considerably (to a tenfold of the initial yeast pitching rate). During bottle refermentation,

yeast growth is much less profound due to the lack of aeration of the medium (i.e. beer) the yeast is pitched in. Due to the minor increase in yeast cell counts, the initial yeast pitching rate during bottle refermentation is more heavily reflected in the final yeast cell counts than during primary fermentation of aerated wort. During bottle refermentation, also the pH was found to effect the final 4VG concentration. At the lowest pH examined (3.76), a substantially lower amount of 4VG was found after the bottle-conditioning step. At this low pH value, yeast cells were less capable of maintaining pH homeostasis than at pH values encountered during wort fermentation.

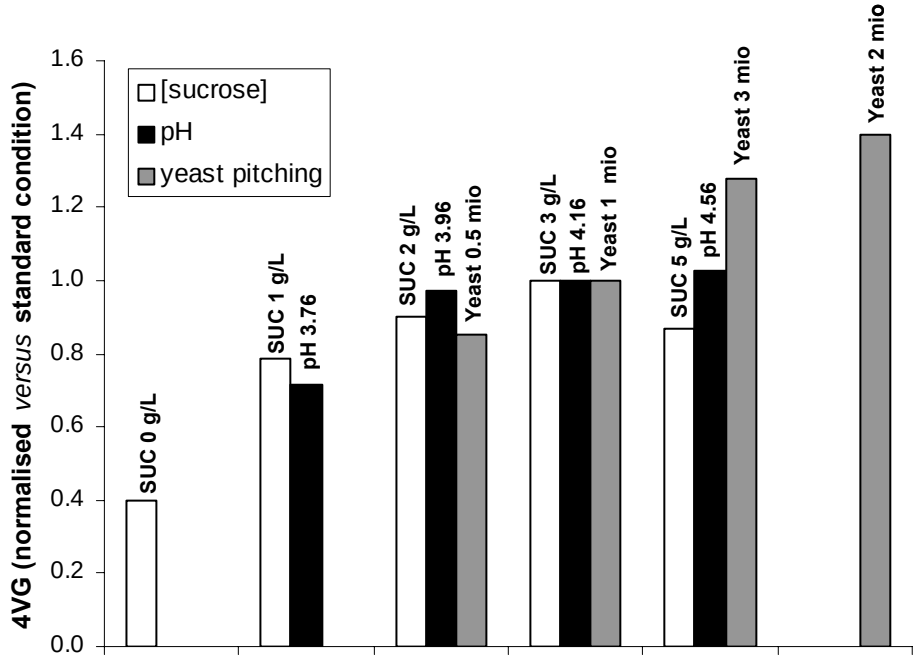


Figure VI.16. *Effect of pH, sugar addition and pitching rate on the formation of 4VG during bottle refermentation – the final amount of 4VG after bottle refermentation is normalised to that found at 3 g/L sucrose, pH 4.16 and 1 million yeast cells*

Also the amount of sugar had a significant effect on the amount of 4VG formed during bottle fermentation. At lower sugar addition levels, final 4VG concentrations increased, while at higher levels the 4VG concentration decreased. Probably the concave curvature (SUC) is caused by an interaction between an increased yeast cell growth counter-acted by an augmented carbon dioxide level at the end of the refermentation process.

VI.3.8. Effect of repitching with top- and bottom-cropped yeast and yeast storage on 4VG formation

Yeast was cropped (top and bottom) from a pilot-scale wort fermentation experiment (paragraph VI.2.7). Top- and bottom-cropped yeast was repitched in wort (14.3 °P, 1.8 L,

EBC tall tube) at 5 x 10^6 cell/mL and incubated at 20 °C. Both yeast suspensions were stored at 0-4 °C for 14 days and repitched a second time in the same wort under the same conditions. Apart from minor differences in fermentation rate, the four fermentations (top/fresh yeast, bottom/fresh yeast, top/stored yeast and bottom/stored yeast) reached final attenuation within 6 days. Final 4VG concentrations were not significantly different between top- or bottom-cropped yeast strains and fresh or stored yeast fermentation experiments (ranging between 1.32 and 1.39 ppm 4VG). Although initial yeast physiology differed, all yeast cultures were able to readily convert the extract. Taken yeast multiplication and the excess of nutrients provided during wort fermentation into account, yeast physiology was probably not much different by the end of fermentation process. It is, however, not possible to extrapolate results through multiple repitching cycles when yeast physiology might deteriorate significantly. When (significant) retardations in fermentation rates are observed, differences in 4VG formation can be expected.

VI.4. CONCLUSION

In the previous chapters, the release of HCA's during wort production has been investigated. These HCA's are flavour-inactive and do not contribute to the flavour of beer. To contribute to the odour and taste pallet of specialty beers, they have to be decarboxylated to the corresponding volatile phenols. The contribution of thermal decarboxylation during wort boiling has already been discussed in chapter V. Volatile phenol concentrations formed during high temperature treatments were not high enough to substantially contribute to the phenolic flavour impression of specialty beers. Hence, the high concentrations often encountered in blond and dark specialty beers must originate from the enzymatic decarboxylation by *Saccharomyces cerevisiae* yeast strains during wort fermentation. In this chapter, the role of brewing yeast strains was characterised. The differences in volatile phenol content in top-fermented specialty beers was reflected in the high incidence of Pad1 phenotype among top-fermenting brewing yeasts strains and the observed differences in Pad1 activity between different brewing strains. More than 70% of the screened top-fermenting yeasts were able to decarboxylate FA. Yeast substrate specificity of the Pad1 enzyme was reflected in the conversion of pCA, FA and SA in commercial beers. It was clearly shown that the majority of 4VG was formed during the second half of the fermentation process when nearly all fermentable sugars were consumed. This suggests that the Pad1 enzyme might be

involved in the course of events triggered upon the stress presented by nutrient depletion. The formation of 4VG during fermentation could be correlated to the Pad1 enzyme activity during fermentation, but not to *PAD1* gene expression levels. This confirmed the results of Clausen *et al.* (1994) and Smit *et al.* (2003) who showed that overexpression of *PAD1* could not be correlated to an elevated enzyme activity and that post-transcriptional regulation is critical for the functioning of Pad1. The temperature and pH dependence, ethanol tolerance and substrate induction of the Pad1 enzyme activity of crude cell extracts or suspensions of intact yeast cells were investigated. Increasing the FA concentration during the propagation phase clearly increased the Pad1 activity of both the crude yeast cell extracts and the whole yeast cell suspensions. The mechanism by which this increase is triggered and whether it is correlated with *PAD1* expression is unknown, but clearly the presence of FA induced Pad1 activity probably to counteract its toxicity. Concerning wort fermentation, the effect of fermentation temperature, fermentation pH, pitching rate, carbon-source and initial FA concentration were investigated. Clearly, when the initial FA concentration increased, so did the final 4VG concentration. This increased decarboxylation rate of FA can be due to both the increased substrate availability and to the substrate induction effects already described. This result is highly relevant, since it validates the possibility of optimising final volatile phenol content in beer by controlling the release of HCA's in the brewhouse. Worts that were supplemented with roasted malt extract exhibited lower levels of 4VG at the end of the fermentation process indicating that Pad1 enzyme activity is affected by roasted malt extract compounds. The observed decrease in the degree of decarboxylation in worts supplemented with specialty malt extracts can explain the observed discrepancy in 4VG content between blond and dark specialty beers. Pilot-scale fermentation experimpents showed that yeast management systems frequently encountered with cilindroconical tank fermentations significantly affect 4VG formation during wort fermentation. Applying counter-pressure decreased both the 4VG concentration and the concentration of flavour-active esters, leading to an unbalanced beer with more pronounced phenolic flavour intensities. Bottom-cropping of yeast in cilindroconical tanks did increase 4VG levels significantly due to the sedimentation of the highly Pad1 active yeast cells through the beer medium capturing and decarboxylating free FA. Bottle refermentation experiments showed that the mechanims applying to it are different from those during primary wort fermentation. Probably due to increased carbon dioxide levels in the fermentation medium, bottle refermentation might not be the most appropriate means to achieve highly flavour-active levels of 4VG in beer. It can, however, be used to optimise final volatile phenol levels in beer.

CHAPTER VII

EVOLUTION OF 4-VINYLGUAIACOL DURING BEER AGEING AND IDENTIFICATION OF A NEW VANILLA-LIKE COMPOUND IN BEER

In this chapter, the decrease in 4VG concentration during beer ageing was examined. The effect of temperature, oxygen and pH on the degradation rate were investigated. A new vanilla-like compound in beer, apocynol, was identified by GC-MS and HPLC-ECD analysis as the main degradation product. In the presence of oxygen, significant amounts of vanillin were formed.

VII.1. INTRODUCTION

McMurrough *et al.* (1996) found that there are appreciable temperature dependent losses of 4VG during the storage of beer. 4VG is transformed to, as yet, unidentified compounds. In wine, the decrease of 4VP and 4VG has partly been attributed to the slow acid-catalysed addition of ethanol on these volatile phenols yielding 4-(1-ethoxyethyl)-phenol and 4-(1-ethoxyethyl)-guaiacol (Dugelay *et al.*, 1995). It has also been suggested that 4VG might undergo oxidation to vanilla-like compounds (Tressl *et al.*, 1976) or oligomerisation. Chassagne *et al.* (2005) have noted the capacity of *S .cerevisiae* yeast lees to sorb 4EG and 4EP. Vinylphenols can also undergo a cycloaddition with anthocyanins yielding red-pigmented pyranoantocyanins (e.g. malvidin-3-*O*-glucoside-4-vinylguaiacol) in wine (Fulcrand *et al.*, 1996; Hakansson *et al.*, 2003; Pozo-Bayon *et al.*, 2004; Wang *et al.*, 2003). In this chapter, the decrease in 4VG concentration during beer ageing is described. To facilitate the detection and identification of novel compounds arising from 4VG during beer storage, forced ageing experiments were conducted in beer model solutions. The presence of the new identified compounds was confirmed in beers that were naturally aged at 20 °C. The effect of temperature, pH and oxygen on the degradation rate and the formation of new compounds were studied.

VII.2. MATERIALS AND METHODS

VII.2.1. Evolution of volatile phenols during beer ageing

Two commercial blond specialty beers produced with Pad1(+) yeast strains with bottle refermentation were aged at 20 °C for 40 weeks. Samples were collected regularly for the quantification of 4VP and 4VG with the HPLC-ECD method described in chapter II. In a second experiment, 2 ppm 4VG was added to a pilsner beer. Before bottle capsulation, the headspace was flushed either with oxygen or carbon dioxide. Samples were incubated at 4, 20, 40 and 60 °C for 12 weeks.

VII.2.2. Forced ageing of 4VG in a beer model medium

A beer model solution was prepared to reflect the concentration of the various species, pH (pH 4.2), nitrogen content and ethanol content (6 % v/v) of beer according to Sadosky *et al.*

(2002). 4VG was added to a final concentration of 3.5 ppm. Samples were forced aged at 60 °C for three weeks. Different experimental set-ups were investigated: the headspace was flushed with oxygen or carbon dioxide, the ethanol content was varied between 5 and 20 % v/v and a broad pH range was examined by setting the pH at 4, 7 and 10. More realistic beer pH's were investigated at pH 3.4, 3.7, 4.0, 4.3 and 4.6.

VII.2.3. Identification of unknown compound by GC-MS and HPLC-ECD

The beer model solution (pH 4; 3 weeks; 60 °C) was extracted three times with ethyl acetate. The combined fractions were evaporated to dryness and redissolved in methanol. Samples were analyses by gas chromatography with mass spectrometry (GC-MS). Samples were injected on a Trace GC Ultra (Thermo, Waltham, MA) equipped with a split/splitless injector, which was used in splitless mode. The carrier gas was helium at a flow rate of 1.5 mL/min. Compounds were separated on a Rtx - 5 Sil MS (60 m x 0.25 mm i.d., 1.0 μm film thickness) (Restek, Bellefonte, PA). The oven temperature profile was: 2 min at 60 °C, to 100 °C at 20 °C/min, to 220 °C at 15 °C/min, to 280 °C at 20°C/min and a final hold of 5 min at 280 °C. Electronic impact mass spectra were recorded at 70 eV (full scan with a mass range from 35 to 350 m/z) on a Dual Stage Quadrupole (DSQ) MS (Thermo, Waltham, MA). A sample of pure reference compound of apocynol was kindly provided by Prof. Ilkka Kilpelainen and Dr. Pirkko Karhunen from the University of Helsinki (Department of Chemistry, Laboratory of Organic Chemistry). They synthesised the apocynol by the reduction of acetovanillone with $NaBH_4$ in 56 % aqueous ethanol as described by Bailey *et al.* (1969). The pure reference compound was analysed by GC-MS and by HPLC-ECD.

VII.2.4. Quantification of apocynol and vanillin in aged beer

Apocynol and vanillin were quantified in aged lager beers, which were supplemented with 4VG according to paragraph VII.2.1 and naturally aged at 20 °C for one year.

VII.3. Results and Discussion

VII.3.1. Evolution of volatile phenols during beer ageing

Two commercial blond specialty beers produced with Pad1(+) yeast strains with bottle refermentation were aged at 20 °C for 40 weeks. Beers were obtained from the breweries

after bottling. Samples were collected regularly for the quantification of 4VP and 4VG. The first beer (6.9 % v/v; 12 EBC, pH 4.41) initially contained 0.61 ppm 4VP and 1.37 ppm 4VG, while the second blond specialty beer (9.3 % v/v; 14 EBC, pH 4.22) initially contained 0.65 ppm 4VP and 2.71 ppm 4VG. The results of the evolution of the 4VG and the 4VP content of both beers during beer ageing are presented in figure VII.1.

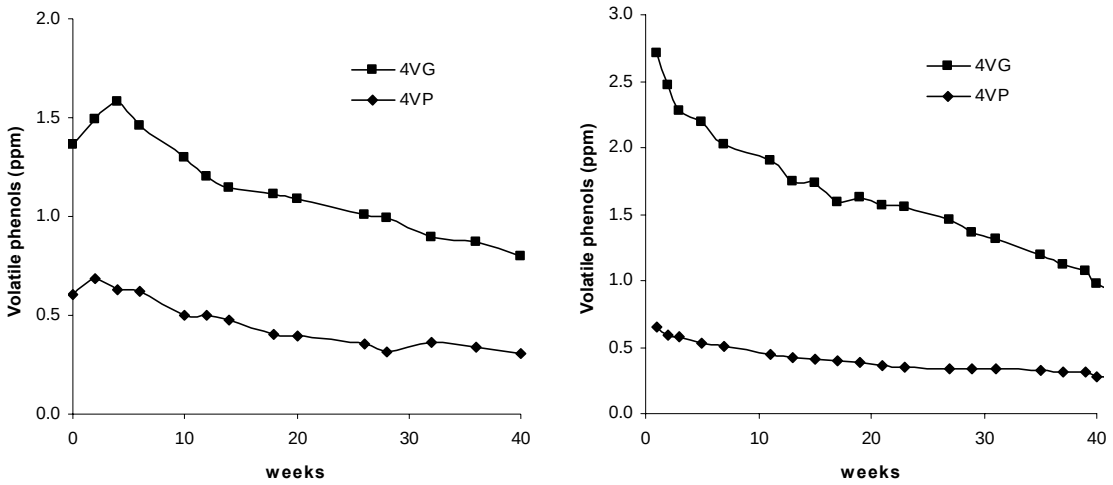


Figure VII.1. *Evolution of 4VP and 4VG during natural beer ageing at 20 °C in two blond specialty beers*

In the first beer, an initial, although small, increase in both 4VP and 4VG content could be noted (0.08 and 0.22 ppm) due to the refermentation with a highly Pad1(+) yeast strain. After the initial increase, the 4VP and 4VG content decreased continuously with a T(1/2) of 34 weeks. In the second beer, the 4VP and 4VG immediately decreased reaching half of the initial value after 31 weeks.

In a second experiment, 2 ppm 4VG was added to a pilsner beer. Before bottle capsulation, the headspace was flushed with oxygen or carbon dioxide. Samples were incubated at 4, 20, 40 and 60 °C for 12 weeks. Results are represented in table VII.1 and table VII.2. As can be seen from table VII.1, the 4VG content of beer decreased more rapidly when the temperature during beer ageing was higher. When the headspace was flushed with oxygen, the final 4VG concentration was significanlty lower than when the headspace was flushed with carbon dioxide (table VII.2). It seems that, at least partly, oxygen might act as a catalysator (radical reactions) or as a reactant. Possible explanations for the decline of the 4VG concentration during beer ageing are: reduction or oxidation of the double bond, oligomerisation, bonding with polyphenols, formation of ethoxyethyl phenols, adsorption to yeast lees or further

metabolisation by yeast. Since 4VG also declined during ageing of the pilsner beer (without bottle refermentation) the latter two cannot solely account for the observed losses of volatile phenols during beer ageing. Moreover, no metabolisation of 4VG by *S. cerevisiae* has been reported so far. When *Brettanomyces* spp. are involved, the loss of 4VG can be due to the reduction of 4VG to 4EG.

Table VII.1. *Evolution of 4VG (ppm) during ageing of a pilsner beer with 2 ppm supplemented 4VG at 4, 20, 40 and 60 °C with carbon dioxide flushed headspace*

	start	**6 weeks**	**12 weeks**
4 °C	2.13	2.11	2.06
20 °C	2.13	1.94	1.74
40 °C	2.13	1.41	1.14
60 °C	2.13	0.66	0.33

Table VII.2. *Evolution of 4VG (ppm) during forced ageing (60 °C) of a pilsner beer with 2 ppm supplemented 4VG and the bottle headspace either flushed with oxygen or carbon dioxide*

	start	**2 weeks**	**6 weeks**	**12 weeks**
60 °C + CO_2	2.13	1.41	0.66	0.33
60 °C + O_2	2.13	0.46	0.33	0.20

VII.3.2. Forced ageing of 4VG in beer model medium

A beer model solution was supplemented with 3.5 ppm 4VG. Samples were forced aged at 60 °C for three weeks. Different experimental set-ups were investigated: the headspace was flushed with oxygen or carbon dioxide; the ethanol content was varied between 5 and 20 % v/v and a broad pH range was examined by setting the pH at 4, 7 and 10. More realistic beer pH's were investigated at pH 3.4, 3.7, 4.0, 4.3 and 4.6. HPLC-ECD chromatograms of the blank sample and those after forced ageing (3 weeks; 60°C) with and without oxygen in the headspace are shown in figure VII.2. The initial blank sample (3.5 ppm 4VG) contained a minor impurity at retention time (RT) 15/16 min (0.15 nA x min). After three weeks at 60 °C, the 4VG concentration in both samples was lower than the initial 4VG content. The sample with the oxygen-flushed headspace contained less 4VG than the sample with the carbon dioxide-flushed headspace (1.90 and 2.36 ppm, respectively).

Runs with varying methanol content in the mobile phase were performed and the run time was varied substantially, but no other substantial peaks could be detected in the model solutions. In both samples, a new peak arose at RT 9 min. The peak area was larger in the

carbon dioxide flushed sample than in the oxygen-flushed sample (1.88 and 1.33 nA x min, respectively). Also the "impurity" peak grew during the forced ageing: 0.18 and 0.54 nA x min for the carbon dioxide and the oxygen sample, respectively. The peak at 15/16 min was identified as vanillin by spiking the sample at various polarities of the mobile phase. The identity was confirmed by GC-MS analysis. Vanillin is one of the possible oxidation products of 4VG. This explains why its concentration in the oxygen-containing sample is higher than in the carbon dioxide flushed sample.

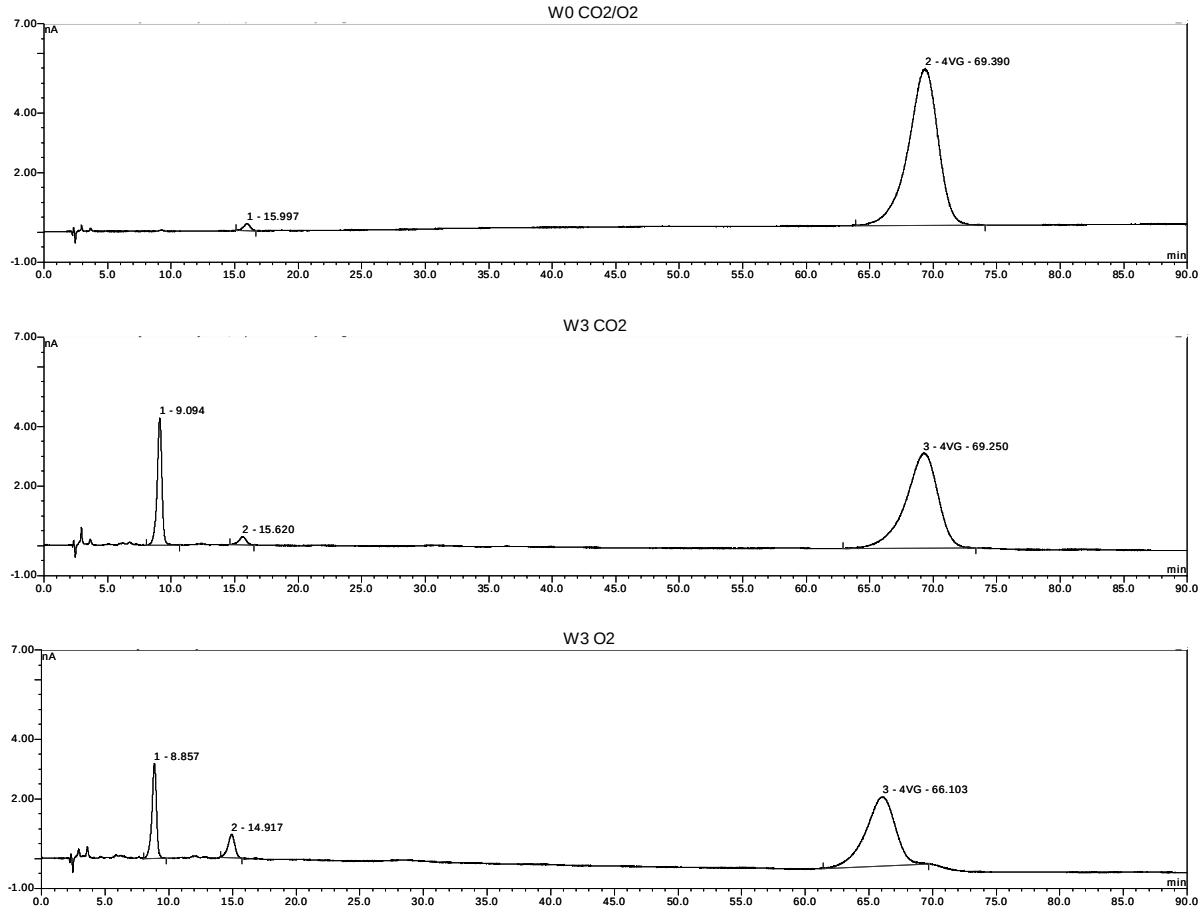


Figure VII.2. *HPLC chromatograms showing the effect of oxygen on the evolution of 4VG and the appearance of new compounds in a beer model solution during forced ageing (60 °C, 3 weeks). From top to bottom: 4VG sample before forced ageing, 4VG sample after forced ageing with carbon dioxide in the headspace and 4VG sample after forced ageing with oxygen in the headspace*

The effect of the pH on the decline of 4VG during forced ageing was investigated at pH 4, 7 and 10. Results are depicted in figure VII.3. The pH had a profound effect on the degradation rate of 4VG. At pH 10, the final 4VG content after forced ageing was the lowest and vanillin was the most abundant compound formed. At pH 7, the final 4VG content remained the highest of the three pH's examined. At pH 4, the final 4VG concentration was lower than at

pH 7, but higher than at pH 10. The most important degradation product formed was the unknown compound at RT 9 min. At higher pH values, the oxidation of 4VG seemed to be the most important route of 4VG decline. The formation of the unknown compound at RT 9 min was likely to be the result of an acid-catalysed reaction. At neutral pH, 4VG was less affected than at high or low pH values.

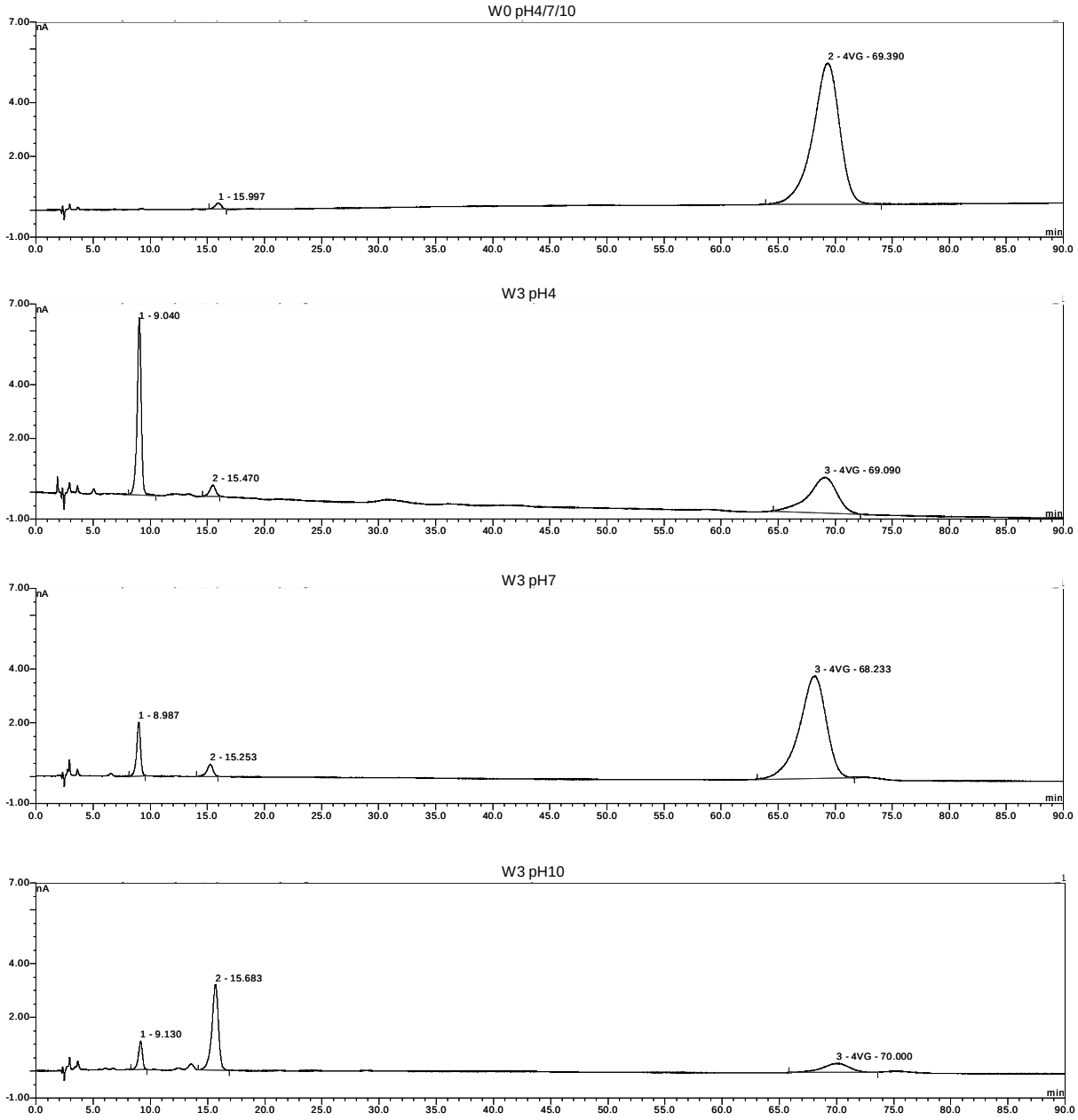


Figure VII.3. *HPLC chromatograms showing the effect of the pH on the evolution of 4VG and the appearance of new compounds in a beer model solution during forced ageing (60 °C, 3 weeks). From top to bottom: 4VG sample before forced ageing, 4VG sample after forced ageing at pH 4, 4VG sample after forced ageing at pH 7 and 4VG sample after forced ageing at pH 10*

The degradation of 4VG was also investigated at more realistic pH's concerning beer ageing (3.4, 3.7, 4.0, 4.3 and 4.6). The results of the 4VG content (ppm), the peak area

corresponding with vanillin (nA x min) and the peak area corresponding to the unknown compound (nA x min) are depicted in figure VII.4. It can be clearly seen that the decline of the 4VG concentration was highly dependent on the pH. Even small changes in pH gave rise to highly differing final 4VG concentrations. The higher final 4VG concentration at higher pH values seemed to be mostly due to the lower amount of the unidentified compound being formed confirming that it must be an acid-catalysed reaction. The formation of vanillin by the oxidation of 4VG increased slighlty with increasing pH values but it was less affected within the examined pH range.

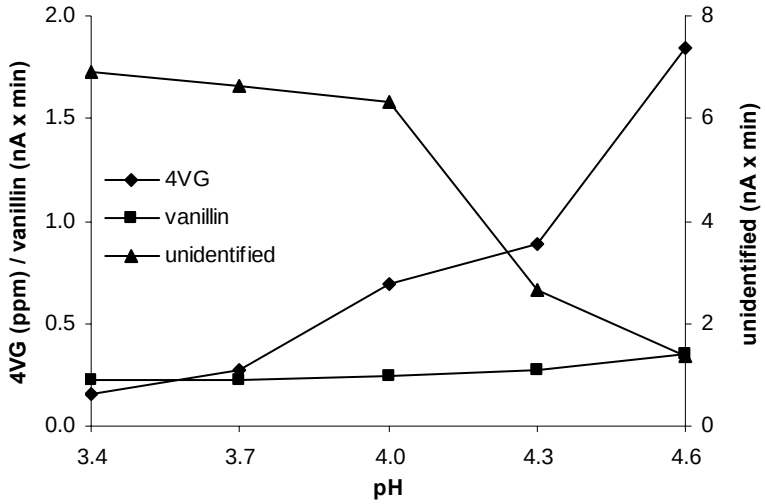


Figure VII.4. *Final amounts of 4VG (ppm), vanillin (nA x min) and unidentified compound (nA x min) after forced ageing (60 °C, 3 weeks) of a beer model solution at different pH's initially containing 3.5 ppm 4VG*

No effect of the ethanol content (between 5 and 20 % v/v) on the degradation of 4VG was observed. This made it unlikely that ethanol would be involved in the decrease of 4VG during beer ageing. Remarkably, when 4VG was forced aged in pure EtOH, no peak at RT 9 min was observed. This confirmed that EtOH was not involved in the formation of the unknown compound.

VII.3.3. Identification of unknown compound by GC-MS and HPLC-ECD

The blank and the aged beer model solutions (pH 4 and 10; 3 weeks; 60 °C) were extracted three times with ethyl acetate. The combined fractions were evaporated to dryness and redissolved in methanol. Samples were analysed by gas chromatography with mass spectrometry (GC-MS). In the aged beer model solution at pH 10, the presence of vanillin was found and could be confirmed by injection with pure reference vanillin and by

comparison with the NIST library. For the unknown compound, the mass spectrum found is shown in figure VII.5. The molecular weight of the compound was 168 leading to the hypothesis that it might have been formed by the reaction of 4VG (MW 150) with water (MW 18). If a hydration of the 4VG molecular structure occurred, it was most likely to occur on the double bond of the vinyl side chain. Hydration reactions are favoured at acidic pH's since the initial rate-limiting step requires the addition of a proton to the less substituted carbon of the double bond. The initial acid-catalysed addition of a proton to the double bond is favoured by the presence of oxygenated electron donating groups on the aromatic ring of 4VG. Moreover, the carbocation formed is stabilised by resonance. In the second step, a water molecule binds to the more highly substituted carbon. The addition follows the Markovnikov's rule implicating that the hydroxyl group binds to the carbon with the highest number of C-C bonds forming the most stable carbocation. The attack of water on the carbocation should gain the compound presented in figure VII.6.

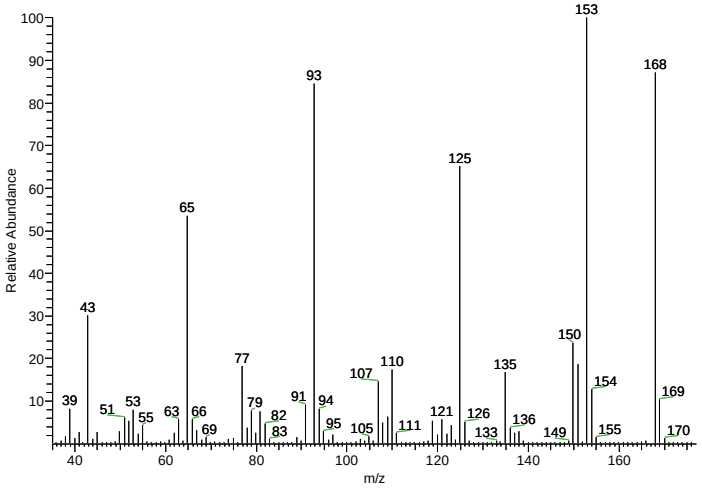


Figure VII.5. *Mass spectrum of the unknown compound obtained by GC-MS electronic impact mode*

The polarity of the suggested product did also correspond with its relative position in the HPLC-ECD chromatograms between the other vanillic compounds. Literature on lignin and wood research, led to the suspect apocynol, also called 1-guaiacylethanol, 4-(1-hydroxyethyl)-2-methoxyphenol, 4-(1-hydroxyethyl)-guaiacol or α-methylvanillyl alcohol. A sample of pure reference compound (obtained by the reduction of acetovanillon) was obtained from the University of Helsinki and confirmed the hypothesis. Both vanillin and apocynol have a clear vanilla-like flavour. A proposed fragmentation pattern of apocynol is shown in figure VII.7. The fragmentation pattern that gives rise to the m/z 125 fragment, is based on the proposed fragmentation pattern characteristic of an hydroxyl group on an

aromatic ring according to McLafferty *et al.* (1992). The mechanism involves the elimination of oxygen with its adjacent ring carbon. The m/z 125 fragment has also been reported in the mass spectrum of vanillyl alcohol according to the NIST library.

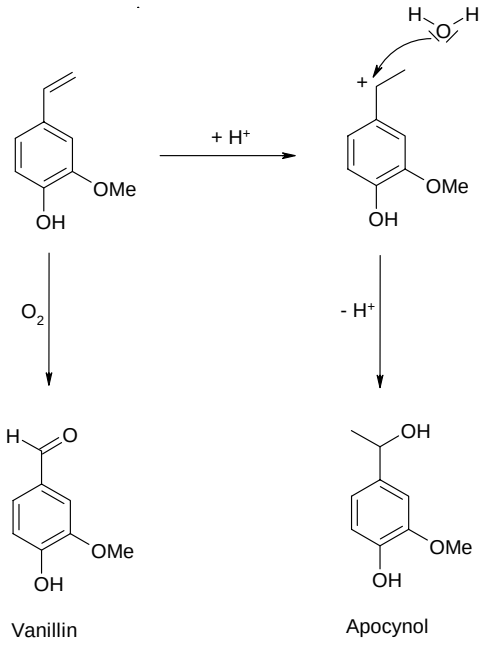


Figure VII.6. *Proposed degradation patterns of 4VG during beer ageing: oxidation leading to vanillin and hydration leading to apocynol*

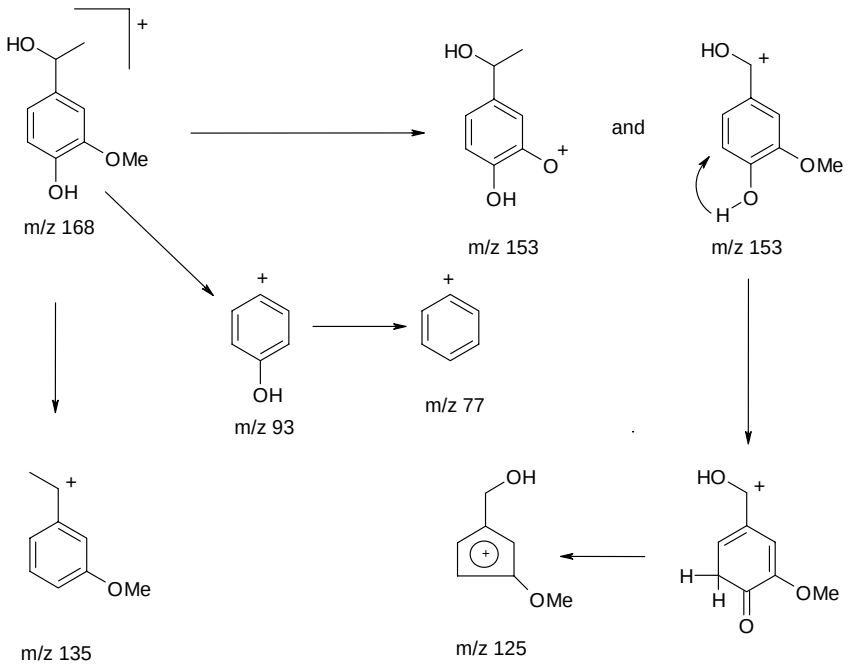


Figure VII.7. *Proposed fragmentation pattern of the molecular ion of apocynol giving rise to the mass spectrum shown in figure VII.5. The fragmentation pattern that gives rise to the m/z 125 fragment is based on the proposed fragmentation characteristic of a phenolic ring moiety according to McLafferty* et al. *(1992)*

VII.3.4. Quantification of apocynol and vanillin in aged beer

Apocynol and vanillin were quantified in aged lager beers, which were supplemented with 2 ppm 4VG according to paragraph VII.2.1. The bottle headspace was flushed with either carbon dioxide or oxygen. Beers were naturally aged at 20 °C and samples were taken after 3, 6 and 12 months for analysis on 4VG, apocynol and vanillin. Values are represented in table VII.3. 4VG decreased more rapidly when the bottle headspace was flushed with oxygen than with carbon dioxide. When the headspace was flushed with carbon dioxide, no vanillin could be detected apart from a small amount (0.04 ppm) in the final sample taken after one year. In contrast, apocynol increased remarkably during the ageing process reaching 0.83 ppm after 12 months. Taken both the amount of vanillin and apocynol into account, more than 85 % of the decrease of 4VG could be explained by the formation of both compounds. When the bottle headspace was flushed with oxygen, both the formation of apocynol and vanillin could be detected. Both accounted for 75 % of the decrease of 4VG during the ageing process. Since oxygen readily forms reactive radical species, it is possible that other oxidation products of 4VG were formed in minor amounts. The reaction of 4VG with oxygen leading to vanillin imparts that 4VG displays AO activity during beer ageing.

Table VII.3. *Evolution of 4VG, apocynol and vanillin (ppm) during ageing (20 °C) of a pilsner beer supplemented with 2 ppm 4VG with and without oxygen in the bottle headspace*

Carbon dioxide-flushed headspace			
	4VG (ppm)	Apocynol (ppm)	Vanillin (ppm)
start	2.13	ND	ND
3 months	1.69	0.36	ND
6 months	1.46	0.44	ND
12 months	1.04	0.83	0.04
Oxygen-flushed headspace			
	4VG (ppm)	Apocynol (ppm)	Vanillin (ppm)
start	2.13	ND	ND
3 months	1.15	0.28	0.33
6 months	0.89	0.37	0.41
12 months	0.31	0.81	0.59

VII.4. CONCLUSION

In this chapter, the evolution of 4VG during beer ageing was investigated and the products, which arise from it, were identified. As already stated by McMurrough *et al.* (1996), there are appreciable temperature dependent losses of 4VG during the storage of beer. Moreover, the degradation rate of 4VG was found to be higher when oxygen was present in the headspace. Experiments in beer model media showed that the loss of 4VG was also highly dependent on the pH, decreasing more rapidly at lower pH's. Hence small increments in beer pH, might severely retard the decrease of 4VG during beer ageing or *vice versa*. Both observations lead to the hypothesis that two reaction mechanism might be involved in the decrease of 4VG during beer ageing. It seemed that, at least partly, oxygen might act as a catalyst (radical reactions) or as a reactant. The other reaction was clearly acid-catalysed. Two new compounds could be detected in beer model media after forced ageing. One of them was identified as vanillin. The concentration of vanillin was higher in the presence of oxygen and increased with increasing pH values. The unidentified compound was formed by an acid-catalysed reaction indicated by the elevated concentrations found at lower pH values. The molecular weight of the unidentified compound was 168 leading to the hypothesis that this compound was the result of a hydration of the double bond of the vinylic side chain of 4VG. Following the Markovnikov's rule, this hydration should lead to a compound called apocynol, which is also called 1-guaiacylethanol, 4-(1-hydroxyethyl)-2-methoxyphenol, 4-(1-hydroxyethyl)-guaiacol or α-methylvanillyl alcohol. It has, to the best of our knowledge, not been reported in beer. Both vanillin and apocynol were quantified in naturally aged lager beers supplemented with 4VG. Apocynol was the main degradation product of 4VG during ageing. In the presence of oxygen, substantial amounts of vanillin were also detected. This indicated that 4VG can act a potential "antioxidant" in beer. Since both apocynol and vanillin have a clear vanilla-like aroma, the decrease of 4VG during beer ageing might impart a shift from a clove-like aroma in fresh specialty beers to a more sweet, vanilla-like flavour impression of aged specialty beers.

GENERAL CONCLUSIONS AND PERSPECTIVES

Phenols in beer are present either in monomeric or in polymeric form. Phenolic monomers in beer include flavonoids such as flavanols (e.g. catechin) and flavonols (e.g. quercetin, myricetin), phenolic acids and volatile phenols. Phenolic acids are simple monocyclic acids and comprise the hydroxy derivatives of benzoic and cinnamic acid. Almost 20 different derivatives of benzoic (e.g. vanillic acid, gallic acid, syringic acid) and cinnamic acid (e.g. *p*-coumaric acid, ferulic acid (FA), sinapic acid, caffeic acid) can be detected in beer. Most of them have high threshold values and do not affect the aroma of beer. However, they are appreciated for their antioxidant activity. Recently, bound phenolic acids also have become a topic of interest because of their potential antioxidant capacity.

Among the **flavour-active volatile phenols,** guaiacol, phenol, vanillin, acetovanillon, eugenol, 4-vinylsyringol, 4-vinylguaiacol and 4-vinylphenol, have been detected in beer. Most of the simple phenolic compounds originate from the raw materials used in the brewing process or from contaminated brewing liquor (e.g. chlorophenols). **Only some of them can be formed by yeast activity, namely 4-vinylguaiacol (4VG) and 4-vinylphenol (4VP)**. The presence of these volatile phenolic compounds is considered undesirable when present in excessive concentration in bottom-fermented pilsner beers, hence the term "phenolic off-flavour" (POF). It is attributed to beers with a strong medicinal, clove-like aroma. Despite being historically catalogued as an off-flavour, these compounds are known to be essential flavour contributors to the characteristic aroma of Belgian white beers (made with unmalted wheat), German Weizen beers (made with malted wheat) and Rauch beers. They also contribute to the aroma of various other alcoholic drinks like whisky and sherry. In wine, 4VP, 4VG and their ethyl analogues (4-ethylphenol and 4-ethylguaiacol) have been detected and are important contributors to wine aroma. Vinylphenols have been also reported as potential off-flavour compounds in non-alcoholic drinks, like apple juice and orange juice, were they impart an old fruit of rotten fruit aroma, and coffee.

In recent years, volatile phenolic flavour compounds have been increasingly encountered in Belgian specialty beers. While some brewers wish for a clear phenolic note in (some of) their beers, others do not aim for their, sometimes overwhelming, presence in the taste pallet of beer. While the origin of volatile phenols in beer basically is known (arising from HCA's extracted from malt, which are subsequently decarboxylated during fermentation) not many means are known for the present day maltster or brewer to optimise them in the final beer. Only few studies have been conducted concerning HCA's in relation to

their flavour potential. The release of the precursors of volatile phenols during mashing has been mostly investigated in the light of their potential antioxidant activity claimed to enhance beer flavour stability or even human health. While some of these results can be extrapolated to the optimisation of the volatile phenol content in beer, little was known of the dynamics behind the release of free HCA's during brewing, the interactive role of native barley enzymes underlying this release and the subsequent decarboxylation to the flavour-active compounds during wort fermentation. Also the causes of the temperature dependent decrease of 4VG during beer ageing needed to be elucidated.

First, a simple and rapid **isocratic RP-HPLC method with amperometric detection** for the simultaneous detection and quantification of hydroxycinnamic acids and their corresponding aroma-active volatile phenols in wort and beer was **developed and validated**. The technique gives good specificity and sensitivity and can therefore be used for routine monitoring of HCA's in wort and beer and the development of volatile phenolic flavour compounds during the beer production process and subsequent ageing. The method is also well suited for the analysis of bound phenolic compounds present in wort and beer after alkaline hydrolysis. The method was used to perform an **extensive survey on the occurrence of HCA's (free and esterbound) and volatile phenols in a range of beer styles**. Odour and flavour thresholds of 4VG were determined in a diverse range of beer styles confirming the contribution of 4VG to the overall flavour perception of many top-fermented specialty beers (especially blond specialty beers and wheat beers). Significant differences in HCA's (both free and esterbound) and volatile phenol concentrations between different beers were observed.

HCA's and volatile phenols were monitored throughout the beer production process to identify realistic control points for the final volatile phenol level in beer. The large variability in HCA's content between different beers, otherwise having likewise properties (original extract content, ethanol content...), suggested that the release of HCA's during mashing might be influenced by various mashing processes and parameters. First, the **variability in the release of free and esterbound HCA's from different malted barley varieties** was investigated. A large variability in HCA's content between different barley malt varieties and their corresponding worts was observed. Differences were also found between free FA levels in worts from identical malt varieties originating from different malthouses. This demonstrates the **importance of selecting a suitable malt variety**. It was shown that only a

small part of the HCA's in malt is transferred to wort during mashing, while the lion's share remains in the spent grains. Free HCA's in wort are both water-extracted and enzymatically released by cinnamoyl esterase activity. Esterase activities clearly differed between different barley malt varieties, as do other AX-degrading enzyme activities. The release of FA during mashing did not only depend on the esterase activity, but also on the amount of esterbound FA released in the wort and on the endoxylanase activity. Concerning the importance of volatile phenols on the flavour perception of many specialty beers, it is advisable that the FA content of Congress wort samples would become included within the malt specifications provided to the brewers. Moreover, it would be interesting to examine the influence of the initial barley quality and the germination and malting process on final HCA's levels in malt and wort more into detail. Probably a lot of the final variation observed in wort can be explained by these parameters.

Apart from the choice of a suitable barley malt variety, **final HCA's concentrations in wort are also seriously affected by brewhouse operations.** A clear difference in temperature and pH dependence between the release of the water-extracted and enzymatically hydrolysed fraction was found. In contrast to the water-extracted fraction, the hydrolysis of esterbound FA is subject to close technological control. An optimal temperature of 40 °C and an optimal pH of 5.8 were found. Also the mash thickness, the grist coarseness and composition, and the stirring regime had a profound effect on the release of FA during mashing. The T,t-dependence of AX degrading enzymes were correlated with free and esterbound FA release during mashing. A pilot-scale (5 hL) wort production process was conducted to validate the results of the laboratory-scale mashing experiments.

HCA's in beer are flavour-inactive and do not contribute to the flavour of beer. To contribute to the odour and taste pallet of specialty beers, they have to be decarboxylated to the corresponding volatile phenols. Concerning thermal decarboxylation in pilsner beer, the combined time of wort boiling, transfer, whirlpool and pasteurisation times can give rise to the 4VG concentrations observed in the survey. However for wheat beers and blond specialty beers, volatile phenol concentrations formed during high temperature treatments were not high enough to substantially contribute to the phenolic flavour impression of specialty beers. Hence, the high concentrations often encountered in blond and dark specialty beers must originate from the **enzymatic decarboxylation of HCA's by *Saccharomyces cerevisiae* yeast strains during wort fermentation**.

The differences in volatile phenol content in top-fermented specialty beers is reflected in the **high incidence of Pad1 phenotype among top-fermenting brewing yeasts strains** and the observed differences in Pad1 activity between different brewing strains. Clearly, the first means for optimising the volatile phenol content in beer is the choice of a suitable yeast strain. During alcohol fermentation, it was clearly shown that the majority of 4VG is formed during the second half of the fermentation process when all fermentable sugars were consumed. This suggests that the Pad1 enzyme might be involved in the course of events triggered upon the stress presented by nutrient depletion. The formation of 4VG during fermentation can be correlated to the Pad1 enzyme activity during fermentation but not with *PAD1* gene expression levels confirming that post-transcriptional regulation is critical for the functioning of Pad1. Clearly, elevated initial FA concentration in wort led to an increased 4VG concentration in beer. This result is highly relevant, since it validates the possibility of optimising final volatile phenol content in beer by controlling the release of HCA's in the brewhouse.

Pilot-scale fermentation experiments showed that **yeast management systems frequently encountered with cilindroconical tank fermentations significantly affect 4VG formation during wort fermentation**. While applying counter-pressure decreased 4VG concentration in beer, so did the concentration of flavour-active esters, leading to an unbalanced beer with more pronounced phenolic flavour intensities. Top-cropping *versus* bottom-cropping of yeast in cilindroconical tanks did increase 4VG levels significantly due to the sedimentation of the highly Pad1 active yeast cells through the beer medium capturing and decarboxylating free FA. Due to the increased carbon dioxide levels in the beer medium, **bottle refermentation** is not a suitable process to achieve highly flavour active levels of 4VG in beer. It can however be used to optimise final volatile phenol levels in beer. Concerning the decarboxylation of HCA's, further investigations should focus on the regulation of yeast's Pad1 activity allowing for better control mechanisms during alcoholic fermentation.

Finally, the **decrease in 4VG during beer ageing** was examined. Two reaction mechanisms leading to the decrease of 4VG during beer ageing were identified. A new vanilla-like compound in beer, **apocynol**, was identified by GC-MS and HPLC-ECD analysis as the main degradation product. Apocynol is formed by an acid-catalysed hydration of the double bond of the vinyl side chain of 4VG. In the presence of oxygen, substantial amounts of **vanillin** were also detected. Since both apocynol and vanillin have a clear vanilla-like aroma, the

decrease of 4VG during beer ageing may impart a shift from a clove-like aroma in a fresh specialty beer to a more sweet, vanilla-like flavour impression of aged specialty beer.

Identification and monitoring of other flavour-active volatile phenols and vanilla-like compounds in beer is becoming increasingly interesting given new state-of-the-art detection methods (such as multiple array electrochemical detection) leading to greater resolution of the complex mixture of aromatic compounds in beer. This should allow for the identification of compounds responsible for other spicy and vanillic flavours in beer.

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