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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

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Abstract

This work represents a survey of the occurrence of hydroxycinnamic acids and volatile phenols in a variety of beer styles. The contribution of 4-vinylguaiacol to the overall flavour perception of top-fermented specialty beers was shown. Significant differences in hydroxycinnamic acids (both free and ester-bound) and volatile phenol content between different beers were observed. The variability in volatile phenol content between different beers and beer styles can be explained by the high incidence of Pad1^{+} phenotype and the variability of Pad1 activity observed among top-fermenting brewing yeast strains. The relative importance of thermal *versus* enzymatic decarboxylation can account for the differences found between bottom and top-fermented beers. Concerning the optimisation of volatile phenol levels in beer, the selection of a suitable brewing yeast strain is the most important means of creating a phenolic taste profile in beer. Given that a considerable amount of hydroxycinnamic acids in beer still occurs in ester-bound form, enhancing the enzymatic release of these phenolic flavour precursors during mashing can greatly enhance the phenolic aroma potential of wort.

Keywords: Volatile phenols; Hydroxycinnamic acids; Wort; Beer; 4-Vinylguaiacol

1. Introduction

Phenols in beer are present either in monomeric or in polymeric form. Phenolic monomers in beer include flavonols, phenolic acids and volatile phenols. Phenolic acids are simple monocyclic acids and embrace the hydroxy derivatives of benzoic (C6–C1) and cinnamic (C6–C3) 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, caffeic acid) can be detected in beer (Floridi, Montanari, Marconi, & Fantozzi, 2003). 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 have become a topic of interest because of their potential antioxidant capacity (Szwajgier, Pielecki, & Targonski, 2005). Beer may be a primary source of bound phenolic acid intake in western diets. However, information about the content of free *versus* bound phenolic acids in beer is scarce (Nardini & Ghiselli, 2004).

Among the flavour-active volatile phenols, guaiacol, phenol, vanillin, acetovanillone, eugenol, 4-vinylsyringol, 4-vinylguaiacol and 4-vinylphenol have been detected in beer (Tressl, Renner, & Apetz, 1976). 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

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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) (Thurston & Tubb, 1981) 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. However, in many other top-fermented blond and dark specialty beers the phenolic flavour is essential for the overall flavour perception.

Volatile phenols have been reported to contribute to the aroma of non-alcoholic beverages like fruit juices and coffee (Donaghy, Kelly, & McKay, 1999; Dorfner, Ferge, Kettrup, Zimmermann, & Yeretzian, 2003; Fallico, Lanza, Maccarone, Asmundo, & Rapisarda, 1996; Marcotte, Stewart, & Fustier, 1998) as well as alcoholic drinks, like beer, wine, sherry and whisky (Chatonnet, Dubourdieu, Boidron, & Lavigne, 1993; McMurrough et al., 1996; Shinohara, Kubodera, & Yanagida, 2000; Tressl et al., 1976; Van Beek & Priest, 2000; Wackerbauer, Kramer, & Siepert, 1982). In wine, the reduction products of 4VP and 4VG have also been detected. These ethyl derivatives originate from vinylphenol reductase activity, typically associated with *Brettanomyces*/*Dekkera* spp. (Chatonnet, Dubourdieu, Boidron, & Pons, 1992; Edlin, Narbad, Dickinson, & Lloyd, 1995). 4VP and 4VG are the decarboxylation products of the phenolic acids *p*-coumaric acid (4-hydroxycinnamic acid) (pCA) and ferulic acid (4-hydroxy-3-methoxycinnamic acid) (FA), respectively (Fig. 1). Phenolic acids (i.e. hydroxycarboxylic acids with phenolic hydroxyl groups), more specifically hydroxycinnamic acids (HCA) 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 in 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 O5. AX are high molecular weight, partly water-soluble polymers. During the brewing process, they are both extracted and solubilised by AX hydrolases from the malt into the wort (Debyser, Derdelinckx, & Delcour, 1997).

FA, pCA and SA can be released as free acids by cinnamoyl esterase activity during mashing. Cinnamoyl esterases have been reported indigenously in barley (Humberstone & Briggs, 2002) and barley malt (Sancho, Bartolome, Gomez-Cordoves, Williamson, & Faulds, 2001; Sun, Faulds, & Bamforth, 2005). Other AX-hydrolyzing enzymes, like xylanase, arabinofuranosidase and xylosidase, are believed to act in synergy with the cinnamoyl esterase in releasing HCA from its bound forms (Sancho et al., 2001). 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: (1) by thermal impact during high temperature treatments in the beer production process, like wort boiling, whirlpool holding and pasteurisation, or (2) by enzymatic decarboxylation during fermentation, by phenylacrylic acid decarboxylase activity of top-fermenting yeasts strains (Pad1 enzyme) (Clausen, Lamb, Megnet, & Doerner, 1994) or contaminating micro-organisms, like *Enterobacteriaceae* (Lindsay & Priest, 1975), lactic acid bacteria (Van Beek & Priest, 2000), acetic acid bacteria and some wild yeasts, like *Brettanomyces*/*Dekkera* spp. (Chatonnet et al., 1992).

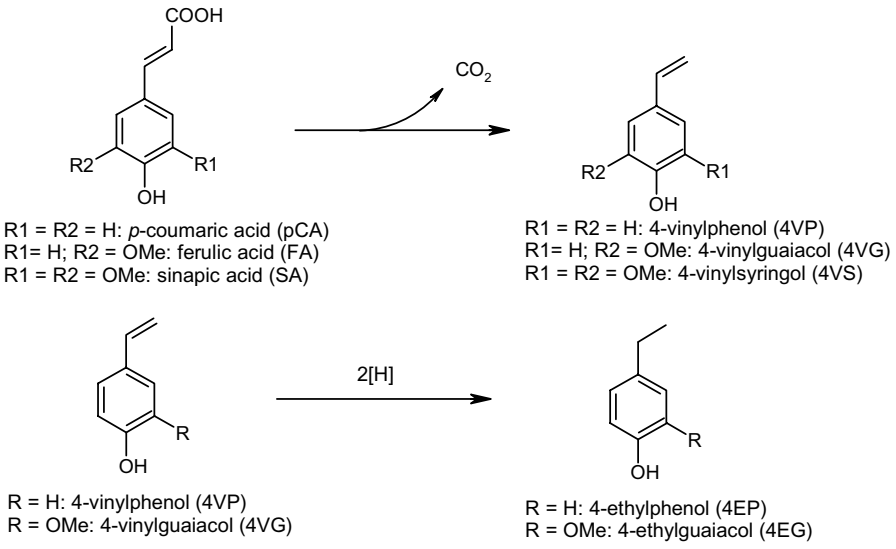

Fig. 1. Decarboxylation of pCA, FA and SA and reduction of 4VP and 4VG to their respective ethyl derivatives.

The objectives of this study were to (1) quantify both free and bound HCA in a variety of beer styles; (2) determine the impact of volatile phenols on the aroma of different beer styles; (3) determine the relative importance of thermal *versus* enzymatic decarboxylation in the formation of volatile phenols during the production of beer; and (4) determine the role of *Brettanomyces/Dekkera* spp. in the formation of ethylphenols in mixed fermentation beers.

2. Experimental

2.1. Materials

All analytes were of analytical grade. pCA, SA, FA, 4-ethylphenol, 4-ethylguaiacol, 4VG and 4VP (10% in propylene glycol) were obtained from Sigma–Aldrich (Bornem, Belgium). 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 made with mixed or spontaneous fermentation (lambic beers, gueuze beers and sour red ales) were obtained from local grocery stores. Pilsner malt was obtained from Cargill Malt Division (Herent, Belgium).

2.2. Quantification of hydroxycinnamic acids and volatile phenols in wort and beer

Quantification of HCA and volatile phenols in wort and beer was performed by HPLC-ECD as described in Vanbeneden, Delvaux, and Delvaux (2006). Before injection, all samples were degassed by sonication for 10 min, filtered through 0.45 μm regenerated cellulose syringe filters (Alltech, Deerfield, IL) into autosampler vials and frozen at −18 °C until analysis. Peak areas were analysed with the Chromeleon Chromatography Management System, version 6.5 (Dionex). All samples were protected from light during analysis to minimise the photo-isomerisation reaction to which HCA are susceptible.

2.3. Determination of total alkali-extractable hydroxycinnamic acid content

For the determination of the total alkali-extractable HCA content in beer, ester-bound HCA 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 glass Pyrex tubes. NaOH solution was supplemented with 1% ascorbic acid (Sigma–Aldrich, Bornem, Belgium) and 10 mM anhydrous 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 4 N HCl and 300 mg NaCl. HCA 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 were resolved in 5.0 ml methanol prior to HPLC 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 $93.6 \pm 4.11\%$, $97.1 \pm 3.64\%$ and $94.2 \pm 5.97\%$ ($n = 3$) for pCA, FA and SA, respectively.

2.4. Determination of odour and flavour threshold of 4VG 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 retronasal), 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 samples of 6 sets of 3 beers, each consisting of two controls and one test sample. Individual best estimate values of threshold are found separately for each assessor as the geometric mean of the highest concentration missed and the next highest adjacent concentration. Group thresholds are then derived from the individual values as the geometric mean of the individual best estimate thresholds. Tasting panels had at least 20 members. The threshold for 4VG was determined 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 their low native 4VG concentrations and as characteristic representatives within their respective beer style, without having noticeable off-flavours. Assessors were asked to describe the flavour perception of the spiked beer samples.

2.5. Thermal decarboxylation

Standard laboratory Congress wort preparations were performed, according to Analytica-EBC (EBC Analytica, 1998, Method 4.5.1). Initially, the wort contained 2.6 ppm FA. After filtration (MN 614, 0.25×32 cm diameter, Macherey-Nagel GmbH), 200 g of filtered wort 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, cooled down and the resulting wort was analysed for 4VG content.

2.6. Screening for Pad1$^+$-phenotype

Saccharomyces cerevisiae brewing yeasts and *Brettanomyces/Dekkera* spp. 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. After semi-anaerobic incubation at 25 °C for 3 days, the supernatant was analysed for volatile phenols by HPLC.

2.7. Yeast propagation

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*g*, 3 min, 4 °C) and resuspended in physiological water (0.9% NaCl). Yeast cell densities were determined microscopically with a Thoma counting chamber.

2.8. Fermentation experiments

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 5×10^6 propagated yeast cells per millilitre 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 and for extract content using a density and sound analyser (DSA-4, A. Paar, Graz, Austria) with a SP-1 autosampler.

2.9. Statistics

Results of 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. The equality of variances (heteroscedasticity *versus* homoscedasticity) was tested with the two-tailed Fisher test. Whenever appropriate, paired analysis was conducted.

3. Results and discussion

3.1. Hydroxycinnamic acids and volatile phenols in commercial beers

Commercial beers belonging to a diverse range of beer styles (bottom-fermented pilsner beers and top-fermented ale beers, Belgian white beers, German Weizenbeers, blond specialty beers and dark specialty beers) were analysed for free HCA and corresponding volatile phenols. The results are summarised in Table 1. Significant differences in HCA 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 3.76 ppm, respectively. Also, 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). This demonstrates a clear difference in Pad1-activity of the yeast strains used for beer production. In each beer, FA was decarboxylated to a larger extent than pCA. 4-Vinylsyringol (4VS), the decarboxylation product from sinapic acid, was not detected with the current method in any beer. 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, Dasnoy, & Collin, 2006). Tressl et al. (1976) have also reported the presence of 4VS in Rauchbeers. Here, the smoke used for the production of smoked malt can be the source as 4VS, a product of the pyrolysis of lignin (Del Rio, Gutierrez, Martinez, & Martinez, 2001).

It can be seen from Table 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 possess Pad1 activity (Coghe, Benoot, Delvaux, Vanderhaegen, & Delvaux, 2004; Perpete, Van Cutsem, Boutte, Colson-Corbisier, & Collin, 2001), volatile phenols in pilsner beers 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 are decarboxylated. Top-fermented beers like Weizenbeers, white beers and blond specialty beers contain significantly more 4VP and 4VG than bottom-fermented pilsner beers. 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 also 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 comprise more than 5% 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.

Table 1
Free hydroxycinnamic acid and volatile phenol content in commercial beers

	$x \pm$ SD[a] (min–max)[b]				
	pCA (ppm)	FA (ppm)	SA (ppm)	4VP (ppm)	4VG (ppm)
Pilsner beer	1.34 ± 0.501	2.00 ± 0.450	0.319 ± 0.082	0.044 ± 0.012	0.138 ± 0.037
($n = 5$)[c]	(0.798–1.94)	(1.32–02.43)	(0.208–0.426)	(0.036–0.065)	(0.087–0.175)
Ale beer	0.924 ± 0.169	1.16 ± 0.804	0.275 ± 0.077	0.308 ± 0.290	0.492 ± 0.482
($n = 5$)	(0.730–1.04)	(0.408–2.36)	(0.141–0.331)	(0.041–0.705)	(0.065–1.08)
Belgian white	0.906 ± 0.431	1.47 ± 0.428	0.393 ± 0.085	0.261 ± 0.139	0.733 ± 0.391
($n = 14$)	(0.348–1.67)	(0.897–2.02)	(0.283–0.516)	(0.055–0.488)	(0.136–1.52)
German Weizen	0.826 ± 0.418	0.814 ± 0.442	0.371 ± 0.150	0.374 ± 0.217	1.11 ± 0.570
($n = 9$)	(0.204–1.34)	(0.354–1.77)	(0.133–0.602)	(0.046–0.846)	(0.165–1.96)
Blond specialty	1.06 ± 0.989	1.25 ± 1.06	0.370 ± 0.180	0.556 ± 0.220	1.38 ± 1.03
($n = 16$)	(0.129–2.77)	(0.071–3.27)	(0.155–0.775)	(0.206–0.963)	(0.470–3.76)
Dark specialty	0.415 ± 0.163	1.07 ± 1.01	0.375 ± 0.243	0.207 ± 0.207	0.514 ± 0.427
($n = 8$)	(0.199–0.597)	(0.094–3.20)	(0.092–0.879)	(0.047–0.663)	(0.053–1.12)
TOTAL	*0.903 ± 0.597*	*1.27 ± 0.808*	*0.364 ± 0.152*	*0.339 ± 0.247*	*0.870 ± 0.758*
(n = 58)	*(0.199–2.770)*	*(0.071–3.27)*	*(0.092–0.879)*	*(0.047–0.963)*	*(0.053–3.76)*

[a] $x \pm$ SD: mean ± standard deviation.
[b] (min–max): concentration range.
[c] n: number of samples analysed.

Total alkali-extractable HCA in beer equal the sum of free and AX ester-bound HCA. The amount of AX ester-bound HCA can be calculated from the amount of total alkali-extractable HCA and the HCA concentration obtained from the analysis of the non-hydrolysed beer samples. Results are expressed in Table 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 originally present in the 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 than 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. Taking into account the higher initial levels of FA in wort and the higher degree of decarboxylation of FA compared to other HCA, 4VG was the most abundant volatile phenol in each beer (Fig. 2). Due to the large variations in HCA contents, no significant differences between pCA, FA or SA content can be found between the different beer styles.

From Table 2 it can be seen that a considerable amount of HCA in beer occurs in AX ester-bound 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

Table 2
Total free (compensated for 4VP and 4VG) and bound alkali-extractable hydroxycinnamic acid content in commercial beers

	$x \pm$ SD[a] (min–max)[b]					
	Total free HCA			Bound HCA		
	pCA + 4VP (ppm)	FA + 4VG (ppm)	SA (ppm)	pCA (ppm)	FA (ppm)	SA (ppm)
Pilsner beer	1.40 ± 0.517	2.18 ± 0.490	0.319 ± 0.082	0.585 ± 0.480	10.1 ± 2.26	1.96 ± 0.333
($n = 5$)[c]	(0.847–2.03)	(1.43–2.61)	(0.208–0.426)	(0.155–1.27)	(7.57–13.8)	(1.58–2.44)
Ale beer	1.33 ± 0.329	1.79 ± 0.481	0.275 ± 0.077	1.04 ± 0.160	11.7 ± 1.28	2.11 ± 0.111
($n = 5$)	(1.05–1.69)	(1.37–2.60)	(0.141–0.331)	(0.926–1.15)	(9.77–13.11)	(1.94–2.19)
Belgian white	1.27 ± 0.378	2.42 ± 0.476	0.393 ± 0.085	1.83 ± 1.10	10.2 ± 1.66	3.68 ± 0.950
($n = 14$)	(0.903–2.06)	(1.54–3.01)	(0.283–0.538)	(0.687–3.43)	(7.25–13.0)	(1.90–5.67)
German Weizen	1.359 ± 0.409	2.24 ± 0.666	0.371 ± 0.150	2.37 ± 0.977	9.80 ± 0.852	3.82 ± 0.781
($n = 9$)	(0.681–1.974)	(1.40–3.30)	(0.133–0.602)	(1.26–4.11)	(8.29–11.1)	(3.04–5.38)
Blond specialty	1.99 ± 1.06	2.77 ± 1.22	0.332 ± 0.149	1.12 ± 0.231	13.6 ± 2.21	2.94 ± 1.01
($n = 16$)	(1.11–3.48)	(1.24–4.85)	(0.155–0.554)	(0.907–1.48)	(9.48–16.3)	(1.59–4.61)
Dark specialty	0.791 ± 0.442	1.82 ± 0.945	0.293 ± 0.153	1.70 ± 0.469	12.0 ± 4.44	2.61 ± 1.43
($n = 8$)	(0.421–1.43)	(0.610–3.27)	(0.092–0.470)	(1.00–2.01)	(9.07–20.8)	(1.62–5.43)
TOTAL	*1.39 ± 0.638*	*2.33 ± 0.858*	*0.344 ± 0.125*	*1.62 ± 0.956*	*11.4 ± 2.62*	*3.08 ± 1.10*
(n = 58)	*(0.421–3.48)*	*(0.610–4.85)*	*(0.092–0.602)*	*(0.155–4.11)*	*(7.25–20.8)*	*(1.58–5.67)*

[a] $x \pm$ SD: mean ± standard deviation.
[b] (min–max): concentration range.
[c] n: number of samples analysed.

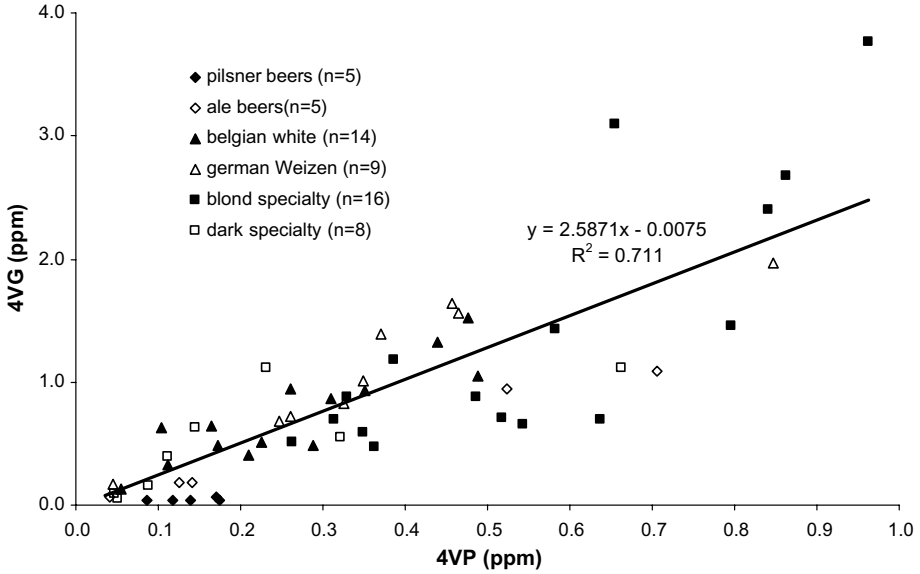

Fig. 2. 4VP *versus* 4VG content in beers belonging to a diverse range of beer styles.

followed by SA and than 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.

3.2. Determination of odour and flavour thresholds of 4VG

The odour and flavour thresholds of 4VG were determined in water and in beers belonging to different beer styles (pilsner beer, blond specialty beer and Belgian white beer). The values in water indicate the *absolute threshold,* i.e. the lowest concentration at which 4VG can be detected. The values for the different beer styles represent the *thresholds of difference* as defined by Meilgaard (1975). This is the smallest difference in concentration, which can be detected. The amount of 4VG pre-existing in beer is hereby ignored. This does not necessarily mean that, at this concentration, the phenolic aroma of 4VG can be recognised as such since the *recognition threshold* may be twice as high as the flavour threshold. Although values may differ between beers belonging to the same beer style, the values offer important information on the overall flavour impact of 4VG. The odour threshold was determined to be 88 ppb, 294 ppb, 400 ppb and 516 ppb for water, pilsner beer, white beer and blond specialty beer, respectively. The flavour threshold was determined to be <20 ppb, 125 ppb, 200 ppb and 367 ppb for water, pilsner beer, white beer and blond specialty beer, respectively. Odour thresholds were higher than the corresponding flavour thresholds because of the limited volatility of volatile phenols and because of the possible dominance of other aroma-active compounds (e.g. esters and sulfur 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) 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 description 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").

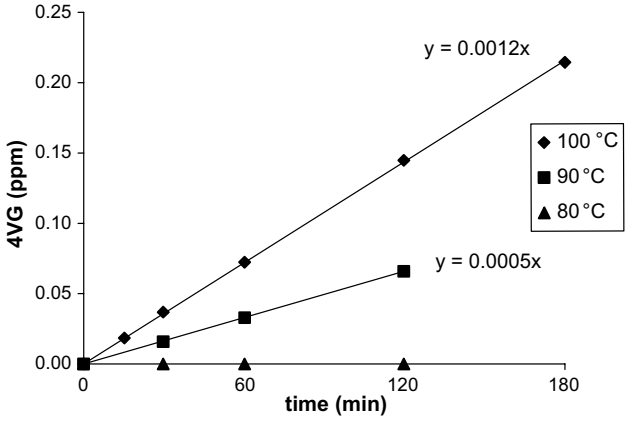

Fig. 3. Effect of thermal load during wort heating on 4VG formation in standard Congress wort.

3.3. Thermal decarboxylation of FA

The formation of 4VG by thermal decarboxylation of FA in Congress pilsner wort is shown in Fig. 3. Mashing temperatures (up to 80 °C) are 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, Parker, Wasserman, and Doerr (1967) noticed that, in dry air, FA only starts to degrade at 200 °C, indicating that the thermal decarboxylation is greatly enhanced under aqueous reaction conditions.

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

$$[4VG]_t = kt + [4VG]_0 \quad (1)$$

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. Here, 4VG may originate from the thermal degradation of FA during the roasting of dark malt (Coghe et al., 2004). The rate constants k (calculated as the slope of the regression line forced through zero) were determined to be 0.0005 and 0.0012 mg/l × min for 90 °C and 100 °C, respectively. The activation energy was calculated from the logarithmic form of the Arrhenius equation ($\ln k = \ln A - E_a/R(1/T)$). From the natural logarithm of the rate constants against the reciprocal of the temperature, activation energy E_a 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.

From the rate constant at 100 °C and the flavour thresholds of 4VG determined in pilsner beer, blond beer and white beer, 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 $1^3/_4$ h, $2^3/_4$ h 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 above.

3.4. Enzymatic decarboxylation of FA

To gain insight into the distribution of Pad1 activity of brewing yeasts, 75 top-fermenting *S. cerevisiae* ale yeasts were screened for their ability to convert FA (10 ml YPD, 100 ppm FA, 3 days, 25 °C, semi-anaerobic incubation). A high incidence of $Pad1^+$ phenotype was observed among top-fermenting brewing yeast strains (Fig. 4). This corresponds 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 top-fermenting yeasts screened 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 and 25% were able to convert between 25% and 50% of the supplemented phenolic acid. Of the studied yeasts 12% were even able

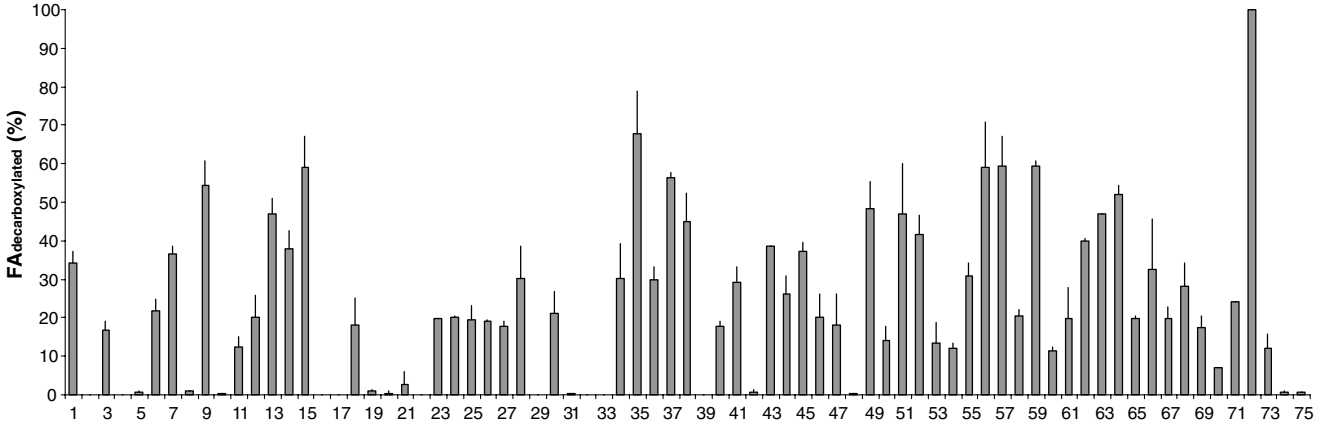

Fig. 4. Decarboxylation of FA (expressed $\%FA_{decarboxylated}$) by 75 industrial top-fermenting *Saccharomyces cerevisiae* brewing strains.

to convert more than half of the FA present in the incubation medium, whereas only 27% 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.

With 11 top-fermenting yeast strains, exhibiting different Pad1-activities during the semi-anaerobic screening procedure, decarboxylase activity was assessed during fermentation of 12 °P wort supplemented with 3 °P glucose and 20 ppm (0.1 mM) FA. Also, during these fermentation experiments, a large variability in Pad1 activity between different yeast strains was observed (Fig. 5). While some yeast strains were unable to convert FA during alcoholic fermentation, others were able to convert all of the added FA within 4 days (corresponding to 15.5 ppm 4VG). The added FA (20 ppm) far exceeds the level of free FA in wort. Moreover, a good correlation was found between the amount of FA being decarboxylated during the semi-anaerobic screening assay and the amount of FA being decarboxylated during alcoholic fermentation on wort medium. Hence the enzymatic decarboxylation of phenolic acids during fermentation can account for the high levels of 4VG often found in top-fermented wheat beers, blond specialty beers and dark specialty beers. In contradiction to what was suggested by Coghe et al. (2004), no yeast strains possessing cinnamoyl esterase activity during fermentation were found.

The *in vivo* substrate specificity of 11 top-fermenting yeast strains was assessed on YPD supplemented with 1 mM pCA, FA and SA. Results are represented in Table 3. 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 fermentation experiments 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.

Table 3
Percentage of pCA, FA and SA decarboxylated by 11 top-fermenting yeasts

	% Decarboxylated		
	pCA	FA	SA
Yeast 1	24	51	0
Yeast 2	12	18	0
Yeast 3	33	56	0
Yeast 4	0	0	0
Yeast 5	10	21	0
Yeast 6	24	27	0
Yeast 7	0	0	0
Yeast 8	0	0	0
Yeast 9	24	28	0
Yeast 10	39	66	0
Yeast 11	29	39	0

3.5. Influence of Brettanomyces/Dekkera spp. on the formation of volatile phenols

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). The free hydroxycinnamic acids 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 3.61 ppm for 4-ethylphenol (4EP) and 4-ethylguaiacol (4EG), respectively. No ethyl derivatives of hydroxycinnamic acids could be detected in normal bottom or top-fermented beers. However, Chatonnet et al. (1992) detected these compounds in wine, in which they have been typically ascribed to the the fermentation and maturation in wooden casks. The

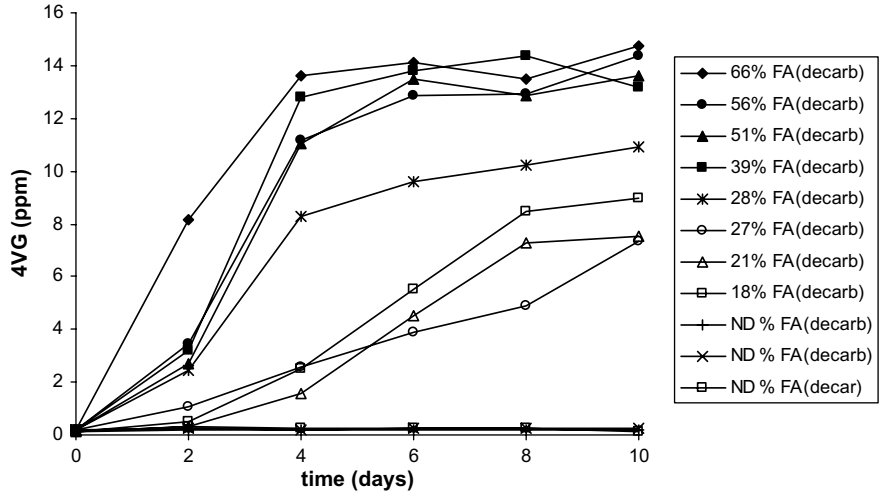

Fig. 5. 4VG formation (ppm) during alcoholic fermentation by 11 top-fermenting yeast strains. The legend expresses the amount of FA being decarboxylated during the semi-anaerobic screening (%FA(decarb)) for Pad1 phenotype on YPD (ND: not detectable).

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 hydroxycinnamic acid is decarboxylated to its vinyl derivative by an enzyme analogous to the Pad1 enzyme of *S 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.

To confirm that the ethylphenols in beers with mixed or spontaneous fermentation could be attributed to the presence of *Brettanomyces*/*Dekkera* sp., 13 subspecies purified from lambic beers (Kumara & Verachtert, 1991; Martens, Iserentant, & Verachtert, 1997) and belonging to the species *bruxellensis*, *custersii*, *anamolus* and *lambicus* were screened for their ability to convert FA to 4VG and 4EG (10 ml YPD, 100 ppm FA, 3 days, 25 °C). All strains were able to convert FA (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 to 4.38 ppm). The hydroxycinnamic acid decarboxylase activity of the *Brettanomyces*/*Dekkera* yeast strains was significantly higher than that of the studied *S. cerevisiae* yeast strains. Notwithstanding, decarboxylation seems to be the rate-limiting step in the formation of ethylphenols.

4. Conclusions

This work represents an extensive survey of the occurrence of HCA and volatile phenols in a variety 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 (both free and ester-bound) and volatile phenol concentrations between different beers were observed. Wort boiling and fermentation experiments, and an extensive survey on the occurrence of the Pad1$^+$ phenotype among top-fermenting brewing yeast strains were conducted to elucidate the origin of the large variability in the volatile phenol concentration between different beers and beer styles. In pilsner beer, given that bottom-fermenting yeasts are Pad1 negative, 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, thermal degradation of FA was found to be of minor importance for the phenolic character of beer. Here, both the high incidence of the Pad1$^+$phenotype and the variability of Pad1 activity observed among top-fermenting brewing yeast strains, found both during semi-anaerobic screening and fermentation experiments, can account for the high levels of 4VG often found in top-fermented wheat beers, blond specialty beers and dark specialty beers and for the large variability of volatile phenol levels observed. The substrate specificity of the Pad1 enzyme is reflected in the conversion of pCA, FA and SA in commercial beers. Concerning the presence of 4EG and 4EP in specialty beers with a mixed or spontaneous fermentation (lambic beers, gueuze beers and sour red ales), the potential of *Brettanomyces* sp. isolated from lambic beers, to convert HCA to their ethyl derivatives was shown.

Although this study was conducted largely as a survey, the information generated can be useful in the understanding of the occurrence of volatile phenols in different beer styles. Concerning the optimisation of volatile phenol levels in beer, the selection of a suitable brewing yeast strain is the most important means of creating a phenolic taste profile in beer. However, the brewers' choice of a yeast strain is mostly dominated by other factors, such as fermentation and flocculation behaviour, overall flavour generation, tradition etc. Hence other means of controlling volatile phenol levels in beer may be necessary. The large variability in HCA content between different beers otherwise having similar properties (original extract content, ethanol content etc.) suggests that the release of HCA during mashing may be influenced by various mashing processes and parameters. McMurrough et al. (1996) already showed the influence of the mashing-in temperature on the release of FA during brewing. Analysis of total alkali-extractable phenolic acids showed that a considerable amount of HCA in beer still occurs in AX ester-bound form. Enhancing the enzymatic release of these phenolic flavour precursors from bound forms during mashing can greatly enhance the phenolic aroma potential of wort. Optimising this precursor release during mashing may be a means of controlling final volatile phenol levels in beer. This will be further investigated in future studies.

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