Characterisation of the Colloidal Haze in Commercial and Pilot Scale Belgian White Beers

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Hazes from four commercial Belgian white beers were studied. Proteins (7-74%) and polyphenols (1.1-7.7%) were the major components, although hazes with a high content of glucose polymers also occurred. The glucose polymers are mainly starch or degraded starch (9-65%) and only to a minor extent β-D-glucan (0-0.5%). Arabinoxylans and metal ions (mainly calcium) were minor constituents. The hazes also contained arabinogalactan-peptide in minor quantities due to the large level of wheat used in the grist. A high mannose content (0.9-3.3%), originating from yeast cell wall mannans, was also found in the hazes.

Characterisation of the haze of three pilot scale brews with different production processes showed that the composition of the hazes was strongly influenced by the process employed. Hazes can be enriched in proteins or starch/dextrins by changing the raw materials and/or the brewing process.

Key Words: *Wheat, beer, haze.*

INTRODUCTION

Belgian white beers are brewed with 60% barley malt and 40% unmalted wheat and top fermented. Following lagering at 15°C, the unfiltered beers are subjected to a second fermentation in the bottle. While in Pilsner beers turbidity is unacceptable, in Belgian white beers both the observed intensity and stability of the haze are quality characteristics. Indeed, consumers expect these beers to have a homogeneous, intense and stable haze. Ironically, it is more difficult to fulfil these expectations than to avoid haze formation.

Haze is caused by the "Tyndall effect", the phenomenon whereby incident light is scattered by microscopic particles in suspension. Because of their dimensions, yeast cells (5-10 µm) contribute to haze. However, the haze caused by yeast is unstable, because during storage it settles down at the bottom of the bottle and this flocculation process is favoured by colder temperatures. Resuspending the yeast (e.g. by shaking the bottle) to restore the haze just prior to consumption is not acceptable to the consumer, because unattractive aggregates are sometimes formed.

Many studies have been carried out on the colloidal haze in yeast-free Pilsner beers[2,3,12,22,26,30,31]. The most frequently encountered haze constituents are proteins and polyphenols. Polysaccharides, metal ions, hop resins and melanodins have also been reported as constituents of Pilsner beer hazes[30]. Although haze formation mechanisms are not fully understood at present, it is generally accepted that interactions between proteins and polyphenols predominate[12]. However, carbohydrates such as starch[23], β-glucans[17,18] and arabinoxylans[5,7] may also play a role in the formation of haze.

It is reasonable to accept that the chemical composition of Belgian white beer hazes differs from that of Pilsner beers since different raw materials (unmalted wheat) and brewing methods, as well as lagering temperatures and a lack of filtration may greatly influence the chemical composition of the haze. The use of unmalted wheat dilutes the content of proanthocyanidins[15], but at the same time increases the level of higher molecular weight proteins, which have been described to cause haze[32].

We have investigated the chemical composition of the non-yeast component of the haze in four commercial white beers. In addition, three pilot scale brews were produced to investigate the influence of both raw materials and brewing process parameters on haze quality and composition.

EXPERIMENTAL

Enzymes

The enzymes used were Pronase E (Merck, Darmstadt, Germany) head stable α-amylase (Termamyl LS 120, Novo

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Nordisk, Bragsvaerd, Denmark) and amyoglucosidase (Boehringer Mannheim, Mannheim, Germany) while Pullulanase, lichenase and β-glucosidase were from Megazyme (Bray, Ireland). The units of enzymic activity specified hereafter are as given by the respective suppliers.

Commercial beers

Four commercial Belgian white beers were examined after filling in 250 ml bottles.

Raw materials

Spring barley malts (Scarlett, Prisma) were supplied by Cargill Malt Division (Herent, Belgium) and Nevada spring malt was from De Wolf-Cosyns (Aalst, Belgium). The wheat used was a mixture of different varieties grown in Belgium. Hallertau (5.6 and 8.5% α-acids, Analytica EBC[1]) and Saaz (3.0% α-acids, Analytica EBC[1]) hops were used for brewing.

Brewing

Three brews (12°Plato) were prepared in a pilot scale brewery[10], with the brewing processes given in Table I. Barley malt (60%, 7.6 kg) and wheat (40%, 5.4 kg) were mashed in 50 litres. The pH of the mash was adjusted with sulphuric acid (1.0 N) and a specific temperature-time profile was followed (Table I). After separation of

TABLE I. Brewing process of three pilot scale brews

	Beer 1	Beer 2	Beer 3
Malt	Scarlett	Prisma	Nevada
Mash pH	5.4	5.6	5.7
Mashing	45 min, 45°C 45 min, 50°C 45 min, 63°C 15 min, 73°C	30 min, 50°C 45 min, 63°C 15 min, 73°C	30 min, 58°C 60 min, 63°C 15 min, 73°C
Filtration temperature	78°C	80°C	90°C
Boiling time	120 min	90 min	60 min

the wort and the spent grains in a traditional lauter tun, the wort was adjusted to pH 5.3, hopped to obtain a bitterness of 15 EBU and boiled. Bitter orange peel and coriander were added 10 min before the end of boiling. The clarified wort was cooled to 22°C, oxygenated and pitched with *Saccharomyces cerevisiae* (5×10^6 cells/ml). The fermentation vessels were kept at 22°C for 10 days and at 15°C for another 10 days. The beer was then transferred in a CO_2 containing keg. After carbonation, the beers were stored at 20°C.

Isolation of haze

Hazes were isolated from four commercial and three pilot beers. Seventeen litres of bottled beer were kept at 0°C for three days to flocculate the yeast. After decantation, the coarse haze (predominantly yeast) was removed by continuous centrifugation at 4,300 g and a flow rate of 6.0 litres/hour (JCF-Z rotor, Beckman, Fullerton, CA, USA) at 15°C. The supernatant was further centrifuged at 37,000 g (JA 14 rotor, Beckman) for 50 min at 15°C to recover the non-yeast component of the haze. The fine sediment was suspended twice in 6% ethanol, centrifuged (48,000 g for 20 min at 15°C; JA 20 rotor, Beckman), frozen with liquid nitrogen and freeze-dried.

Analysis of malts and beers

Malt and beer analyses were performed according to Analytica EBC[1]. Total polyphenols in the malt were determined in the Congress worts. Arabinoxylans were determined as described by Debyser *et al*[8] and expressed as xylans (% xylose x 0.88). Haze were determined nephelometrically at 15°C with a Haffmans (Venlo, The Netherlands) nephelometer (range 0-20 EBC or 0-100 EBC) and expressed in EBC units[1]. Foam stability was measured with a NIBEM foam stability tester supplied by Haffmans.

Haze analysis

Microscopic analysis

Isolated haze (fine and coarse) was placed on a microscope slide, covered with a cover slide and examined with a Leitz Laborluk K microscope (x 600 magnification, Ernst Leitz Wetslar GMBH, Wetzhal, Germany). It was stained with iodine or eosin yellow before microscopic examination, to determine the presence of dextrin/starch or proteinaceous matter respectively[5].

Protein

The protein level (N x 6.0) was determined in the freeze-dried haze using a micro-Kjeldahl method[20].

Polyphenols

Total polyphenols were determined according to Jerumanis[1] with some modifications. Instead of 10.0 ml beer, 10.0 mg freeze-dried haze suspended in 10.0 ml water was used. After addition of the ferric and the ammonia reagents, the coloured suspension was centrifuged (10 min, 48,000 g, 20°C) and the absorbance measured at 600 nm.

Monosaccharide composition

The neutral sugar composition was determined by gas chromatography of alditol acetates obtained after hydrolysis, reduction and derivatisation by Englyst and Cummings[11]. The derivatives were separated on a Supelco (Bellefonte, PA, USA) SP-2380 column (30 m, 0.32 µm ID, 0.2 mm film thickness) in a Chrompack 9011 Chromatograph (Middelburg, The Netherlands) equipped

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with a flame ionisation detector. The carrier gas was He. Separation was at 225°C, with injection and detection temperatures of 275°C. The internal standard was β-D-allose.

Starch

Freeze-dried haze (15.0 mg) was suspended in 2.0 ml sodium acetate buffer (0.2 M, pH 5.0) and boiled for 5 min. Pronase E [300 units in 0.5 ml sodium acetate buffer (0.2 M, pH 5.0)] was added and the mixture kept at 37.5°C for 20 hour. After boiling for 5 min and cooling to 40°C, pullulanase [7 units in 10 µl sodium acetate buffer (0.2 M, pH 5.0)] was added and the mixture incubated at 40°C for 8 hours. Finally, amyloglucosidase [30 units in 0.1 ml sodium acetate buffer (0.2 M, pH 5.0)] was added and the mixture held at 60°C for 8 hours. After boiling (5 min) and freeze-drying, free glucose was determined by gas chromatography as described above, omitting the hydrolysis step.

β-glucans

Freeze-dried material (15.0 mg) was suspended in 2.0 ml sodium acetate buffer (0.2 M, pH 6.0) and boiled for 5 min. After adding Pronase E [300 units in 0.5 ml sodium acetate buffer (0.2 M, pH 6.0)], the mixture was kept at 37.5°C (20 hours) and subsequently boiled for 5 min. After treatment with 0.1 ml lichenase (0.2 units, 1 hour, 40°C) and 0.5 ml β-glucosidase (0.04 units, 1 hour, 40°C), it was boiled for 5 min, lyophilised and free glucose was determined as above.

Determination of uronic acids

Uronic acids were determined colorimetrically by the method of Englyst and Cummings[11] with galacturonic acid as standard. After hydrolysis with sulphuric acid (2.0 N) the mixture was filtered, the dimethylphenol reagent added and the extinctions at 400 and 450 mm measured.

Determination of metal ions

Freeze-dried material (40.0 mg) was ashed (2 hours, 600°C) in a pre-ashed (2 hours, 600°C) crucible. The ash was solubilised in 4.0 ml 5.0 M HCl containing 1% La_2O_3. The crucible was washed two times with 3.0 ml 5.0 M HCl (containing 1% La_2O_3). The solutions were combined and adjusted to 20.0 ml with deionised water and transferred over an ash-free filter (Whatman, nr 44). Metal ions (Ca, Cu, Fe, Mg, Zn) were determined by atomic absorption spectrometry (model IL 651, Instrumentation Laboratory Inc, Wilmington, MA, USA).

Determination of water content

Water levels in freeze-dried haze were estimated with a 701 KF Titrino (Metrohm, Herisau, Switzerland), based on the Karl Fisher method[29].

Defatting of samples

Hazes were defatted as described by Roels *et al*[28]. Samples (15.0 mg) were extracted with two subsequent portions of 2.0 ml water saturated n-butanol for 1 hour at 100°C with frequent intermediary shaking. The samples were centrifuged for 10 min at 1,250 g (20°C), the residue was washed with 2.0 ml ethanol and centrifuged (10 min at 1,250 g). After washing with acetone and diethyl ether they were allowed to dry overnight at 30°C.

All analyses described above were carried out at least in duplicate and the mean values are presented. The coefficient of variation (CV) of the results was smaller than 8%, except for the enzymatic determination of starch and β-glucans (CV<15%).

RESULTS AND DISCUSSION

Pilot scale brewing

Experiments were designed to determine the impact of raw materials and brewing process parameters, and therefore the composition of the beers, on the colloidal haze (Table I). Different malts (Table II) were used. For the first brew, a well modified malt with a high Kolbach index, a strong proteolysis during brewing and an intense boiling programme were chosen. For the third brew, the opposite conditions, a poorly modified malt with a low Kolbach index, a limited proteolysis during brewing and an intense boiling programme, were used. It was anticipated that the latter conditions would lead to more "haze active" (high molecular) proteins in the beer[9,13]. The second brew was made with intermediate conditions.

TABLE II. Analysis of Scarlett, Prisma and Nevada malts

Analysis	Scarlett	Prisma	Nevada
Moisture content (%)	4.5	4.3	5.6
Extract on dry malt (%)	84.1	81.8	79.8
Fine-coarse difference (%)	1.0	1.3	2.4
Wort colour (EBC units)	3.2	4.5	3.2
Total protein on dry malt (%)	10.0	10.4	9.1
Soluble protein on dry malt (%)	4.9	4.3	3.6
Kolbach index (%)	49	41	39
Viscosity (cP)	1.56	1.58	1.59
Total polyphenols (mg/litre)	51	65	42

The sparging temperature was also changed because this influences the amount of high molecular starch/degraded starch products[21].

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Beers: composition

Table III lists the analytical data of both the commercial and the experimental beers. The composition of the commercial beers was consistent with what is found in the marketplace. The original extract was ca. 12° Plato, although the apparent extract and, therefore, the degree of attenuation and the ethanol concentration can be different. Bitterness and colour readings were low. Although none of these beers were subjected to a colloidal stabilisation treatment, their polyphenol levels were low. This can mainly be ascribed to the low wheat polyphenol content[4].

The major difference between the experimental beers was the protein level. Beer 1 contained 4.4 g/litre while beer 3 contained only 3.2 g/litre. This is due to different Kolbach indices and protein levels in the used malts, as well as to the mashing conditions (Table I). The high mashing temperatures and the high pH used to produce beer 3 are unfavourable for proteolysis. Low levels of proteins were therefore solubilised[9].

Because the majority of the parameters of the experimental and the commercial beers were quite comparable, it is reasonable to accept that the experimental brewing process is representative for industrial brewing. The only major difference was the amount of air in the bottle, which, of course, may have an effect on the haze[16].

The haze quantity and microscopic evaluation

Haze intensity depends on temperature and the haze formed at 0°C which redissolves at 20°C is called chill haze. The haze remaining at, or above, 20°C is defined as permanent haze. Hazes formed at an intermediate temperature of 15°C were studied.

Figure 1 shows there is a linear relationship ($R^2 = 0.86$; $n = 7$; $P = 0.003$) between the amount of haze (mg dry matter/litre) recovered from the beers centrifuged at low g (4,300 g, 0°C) by renewed centrifugation at high g values (37,000 g, 15°C) and the haze determined nephelometrically at 15°C. This suggests that the haze collected by centrifugation is representative of that present in the beers. Haze levels (dry matter) differed between 18.5 and 40.1 mg/litre. Although different brewing processes were used for the experimental beers, the beers contained comparable amounts of haze. In

TABLE III. Beer analyses of commercial (A-D) and pilot scale (1-3) Belgian white beers

	Beer A	Beer B	Beer C	Beer D	Beer 1	Beer 2	Beer 3
Original extract (°Plato)	11.7	12.1	12.0	12.1	12.0	11.8	11.8
Final extract (°Plato)	4.2	4.7	3.9	3.7	4.6	5.2	4.7
Alcohol (% vol/vol)	4.9	4.9	5.4	5.5	4.9	4.3	4.7
Real attenuation (%)	64	61	68	70	62	56	60
Bitterness (EB Units)	8.9	8.2	13	13	14	17	13
pH	4.3	4.1	3.9	4.2	4.3	4.4	4.0
Colour (EBC Units)	6.5	6.0	6.0	7.5	8.0	8.5	6.5
CO^2 (g/litre)	6.2	5.8	5.2	5.7	4.9	5.1	5.2
Air in bottle (ml/bottle)	6.0	0.6	0.7	0.9	0.2	0.2	0.3
Haze (EBC Units, 15°C)	27	39	8.9	18	16	16	24
Foam stability[a] (s/3 cm)	248	272	191	200	186	203	211
Viscosity (cP)	1.61	1.78	1.53	1.79	1.59	1.56	1.70
Proteins (g/litre)	4.2	4.0	3.1	4.0	4.4	4.3	3.2
Polyphenols (mg/litre)	101	158	103	136	124	146	149
Xylans[b] (mg/litre)	877	973	885	1010	1304	1124	1209
Beta-glucans (mg/litre)	83	209	68	200	87	91	92

[a]: Foam stability tested by the NIBEM method;
[b]: Xylans expressed as % xylose x 0.88.

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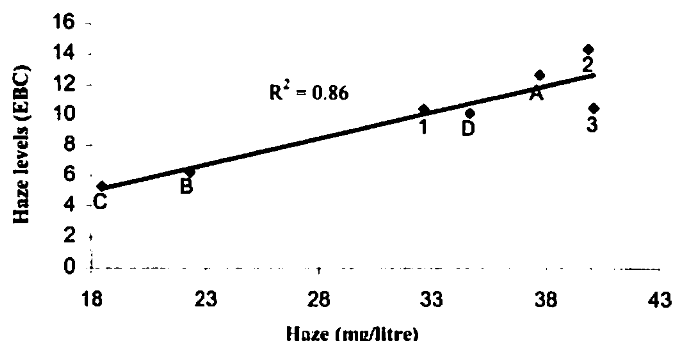

FIG. 1. Relationship between the haze intensity determined nephelometrically in beer samples and the dry haze (mg/litre) recovered from beers; Beers A–D = commercial beers; beers 1–3 = experimental beers.

most of the beers, the nephelometrically determined haze of the uncentrifuged beers (Table III) differed significantly from the haze in the centrifuged (4,300 g) beers (Fig. 1). Microscopic analysis of the coarse haze (isolated at 4,300 g) showed that it consisted mainly of yeast. In some cases, oxalate crystals could be recognised by light microscopy.

No yeast or other micro-organisms were present in the fine haze. In all samples, amorphous material and small spherical particles were observed. Staining with iodine or eosin yellow indicated that all hazes consisted mainly of dextrin and proteinaceous matter. The hazes of beers C, D and 3 stained deep blue with the iodine reagent, indicating that they contained a substantial concentration of dextrin/starch with a degree of polymerisation of at least 47[19].

Chemical composition of the haze from commercial beers

The three main components of haze isolated from four commercial white beers were proteins, polyphenols and carbohydrates as shown in Table IV. The contents of proteins and polyphenols varied between 6.9 and 73.9% and between 1.1 and 7.7% respectively (Table IV). It seems that low protein contents of the beers (Table III) go hand in hand with the levels of proteins in the haze.

The carbohydrate contents varied between 18.6 and 94.5% (Table IV). Polymers of glucose, accounting for

TABLE IV. The amount and the chemical composition of the hazes in commercial (A–D) and pilot scale (1–3) Belgian white beers

	Beer A	Beer B	Beer C	Beer D	Beer 1	Beer 2	Beer 3
Haze (mg/litre)	38	22	18	34	33	40	40
Proteins (%)	**72.4**	**73.9**	**6.9**	**35.5**	**79.7**	**76.7**	**30.3**
Polyphenols (%)	**4.3**	**7.7**	**1.1**	**2.8**	**4.9**	**10.3**	**3.3**
Carbohydrates[a] (%)	**21.1**	**18.6**	**94.5**	**63.2**	**14.3**	**17.2**	**62.0**
Glucose (%) Starch	10.3	10.0	72.1	49.7	2.3	6.5	57.9
β-glucan	0.5	0.0	0.5	0.6	0.3	0.3	0.4
Residual	7.9	6.1	28.4	16.0	4.3	5.2	6.2
Arabinose (%)	0.73	0.94	1.08	0.71	2.24	1.51	0.88
Xylose (%)	0.90	0.76	0.66	0.64	1.39	1.06	1.02
Galactose (%)	0.80	0.84	1.28	0.88	2.21	1.38	0.66
Manose (%)	2.56	2.00	0.99	2.18	3.20	3.34	1.24
Metal ions (%)	**0.63**	**0.18**	**0.21**	**0.51**	**0.19**	**0.79**	**0.08**
Iron (‰)	0.56	0.30	0.39	0.57	0.24	0.47	0.03
Calcium (‰)	3.97	0.93	1.19	3.51	1.18	6.59	0.47
Copper (‰)	0.37	0.14	0.15	0.33	0.29	0.29	0.15
Zinc (‰)	1.02	0.11	0.13	0.31	0.05	0.07	0.03
Magnesium (‰)	0.33	0.20	0.26	0.41	0.18	0.50	0.13
Uronic acids (%)	**0.82**	**1.04**	**0.65**	**0.40**	**2.23**	**1.52**	**0.71**
Total	**99.3**	**101.4**	**103.4**	**102.2**	**101.3**	**106.5**	**96.4**

[a]: Carbohydrates = sum of the monomers * 0.9.

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14.5 to 90.9% of the hazes, were by far the predominant polysaccharides. Between 54 and 75% of the glucose stemmed from starch and between 0.0 to 2.2% originated from β-glucan. The presence of starch and/or degraded starch in haze is not surprising, because the use of 40% unmalted wheat in the brewing process can result in "blue mashes" (iodine test) after filtration[4]. The residual glucose probably originated from dextrins/starch or β-glucans that could not be completely hydrolysed enzymically to glucose monomers and consequently could not be detected.

Relatively high levels of mannose (0.9-3.3%) were found. Siebert *et al*[31] suggested earlier that mannose in beer hazes originates from yeast cell wall mannans. These mannans are the major components of the yeast exocellular polysaccharides. They can be released during fermentation at high temperatures and/or extended storage over lees[24]. The high temperatures during fermentation (23-25°C), lagering (15°C) and re-fermentation in the bottle, followed by a storage period in the presence of yeast can obviously lead to large levels of mannans in white beers. The level of mannose in white beers, as determined by gas chromatography[11], was twice as high as that in Pilsner beers (results not shown).

Arabinose (0.7-1.1%), xylose (0.6-0.8%) and galactose (0.4-1.3%) were minor components of haze. Most xylose and part of the arabinose most likely originated from arabinoxylans. Different researchers have shown that arabinoxylans can indeed contribute to hazes[5,7] and Buckee[5] has suggested that the arabinoxylans of the haze originated from wheat. To examine whether galactose is associated with glycolipids or arabinogalactan peptides, the samples were defatted. Following defatting, the galactose contents of the samples remained unchanged. It can therefore be assumed that galactose is associated with arabinogalactan-peptides. The fact that rather high arabinogalactan-peptide contents can be found in white beers may not be so unlogical since Loosveld *et al*[25] have shown that these components are associated with wheat. Although unexpected, however, this work for the first time demonstrated that these water soluble components are part of beer hazes. The fact that more arabinogalactan-peptides than arabinoxylans were found in the hazes, although the level of arabinoxylans was much higher in beers, suggests that arabinogalactan-peptides interact more easily with haze proteins than arabinoxylans. In this context, it is of note that Roels *et al*[28] earlier found that during wheat gluten coagulation arabinogalactan-peptides are entrapped in gluten relatively more than water-extractable arabinoxylans.

The variability of the haze mineral composition (Table IV) also indicates the heterogeneous character of hazes in white beers. Although the levels of the metal ions were very low (except for calcium), their impact on haze formation may not be neglected[6]. It is known that calcium can form crystals with oxalic acid released by the yeast. In the coarse haze of beers A and D, calcium oxalate crystals could be visualised by light microscopy. High contents of calcium ions were also present in the fine haze of these beers, although no crystals could be observed in these hazes.

Chemical composition of the haze from experimental white beers

As mentioned above, haze concentrations in the experimental beers were comparable. Their composition, however, was quite different (Table IV). Whereas proteins were the major components in the haze of beer 1 (79.7%), the haze of beer 3 contained mainly carbohydrates (62.0%).

When comparing the concentrations of insoluble proteins in the experimental beers in more detail, it became clear that they were influenced by the malting and brewing process. Although the level of polyphenols in the beers was the same (Table III), different quantities of proteins were present in the hazes. In the brewing literature[9,13], it is reported that more extensively degraded proteins are less susceptible to precipitation. Thus, the use of adequately modified malts, combined with mashing conditions favouring proteolysis, gives rise to a smaller amount of haze sensitive proteins[9,13,14]. This may explain why slightly less proteins were precipitated in beer 1 (79.7% of 33 mg haze/litre), brewed with well-modified malt and a long protein rest at pH 5.4 than in beer 2 (76.7% of 40 mg haze/litre).

However, since the level of protein in the haze of beer 3 was very low, a malt with a poor modification and no protein rest during brewing did not lead to a protein rich haze. It seems that under these conditions the quantities of haze sensitive proteins solubilised during malting and brewing were very low.

While the hazes of beer 1 and beer 2 contained a substantial amount of glucose, mainly originating from starch and/or dextrin, glucose was the major component (more than 60%) of the haze in beer 3. This high level of glucose could mainly be due to the high sparging temperature at 90°C. This observation is in agreement with Kunze[21] who stated that undisolved residual starch is washed out of the spent grains during sparging. When the sparging temperature was above 78°C no late saccharification occurred, resulting in "blue mashes".

As in the hazes of the commercial beers, arabinogalactan-peptides, arabinoxylans, β-D-glucans, glucomannans and metal ions were minor components of the hazes of the experimental beers. It is significant that protein rich hazes contained more of these

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components than the starch rich haze. This could point out that these components together with proteins, exerted a co-precipitation behaviour.

CONCLUSIONS

The chemical composition of hazes isolated from four commercial and three experimental Belgian white beers was characterised. In all isolated hazes, major components were protein-polyphenol complexes and starch/degraded starch products. Minor carbohydrate components detected were arabinoxylans, arabinogalactan-peptides, mannans and β-D-glucans. Analysis of the metal ion composition showed that calcium was the most abundant ion present and that it probably originated from calcium oxalate crystals.

Furthermore, the influence of the raw materials and brewing conditions on haze formation and chemical composition was investigated. Under the experimental brewing conditions, the haze quantity was comparable although the chemical composition could be significantly altered. Thus, by changing the degree of malt modification and the brewing process (pH of mashing, temperature-time profile, sparging temperature and boiling time), hazes rich in protein or starch/degraded starch products were obtained.

This information clearly shows that it is possible to control the chemical composition of the haze in Belgian white beers and thus to obtain hazes low in starch/starch degraded products which have been reported[27] to lead to an unstable product, unacceptable to the consumer.

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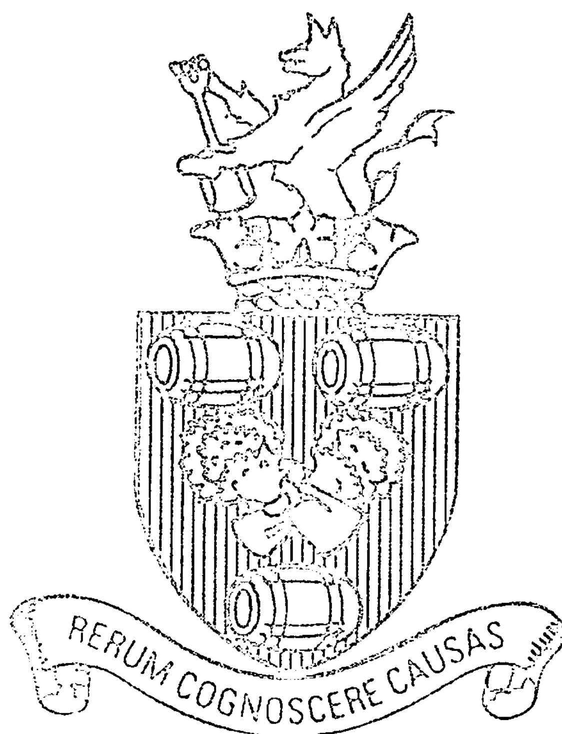

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