

# Beer Fluorescence and the Isolation, Characterisation and Silica Adsorption of Haze-Active Beer Proteins

Kathleen Apperson,<sup>1</sup> Kenneth A. Leiper,<sup>2</sup> Ian P. McKeown,<sup>3</sup> and David J.S. Birch<sup>1,4</sup>

## ABSTRACT

**J. Inst. Brew. 108(2), 193–199, 2002**

The fluorescence spectra and lifetimes of diluted beer have been explored and found not to report on protein removal either by silica or tannic acid, nor polyphenol uptake by PVPP. Comparing the fluorescence spectra of beer with that of tea and hops, it seems that proteins, complex polyphenols and iso- $\alpha$ -acids can contribute to the intrinsic fluorescence of beer, although the contribution from polyphenols must be minimal since treatment with PVPP does not dramatically change the background fluorescence. To eliminate the problem of background fluorescence haze-active protein was isolated. Steady-state and time-resolved fluorescence techniques were used to characterise these and to monitor their uptake by different silica gels as a function of pH. Heat treated large pore volume, small surface area silicas were the more effective adsorbers for the proteins under study, with pH 4 being optimum. Using both intrinsic amino acid fluorescence and the extrinsic fluorophore fluorescamine, the time-resolved fluorescence anisotropy has been measured and the radius of the isolated haze protein found to be  $\sim 35$  Å. Comparisons have been made with proteins of known size and structure such as human and bovine serum albumins (HSA and BSA).

**Key words:** Beer, fluorescence, haze-active protein, polyphenols, silica adsorption.

## INTRODUCTION

The intrinsic fluorescence characteristics of beer are expected to be complex due to the overlap of emissions from numerous species. Nevertheless once the different origins of the fluorescence have been identified, by comparison with the isolated species, new insights may be found into the brewing and stabilisation processes. With this goal in mind, various constituents in beer have been studied and compared using steady-state and time-resolved fluorescence techniques.

The interaction of protein with amorphous silicas, eg., silica hydrogels, silica xerogels, is of interest to the beverage industry.

Of importance is its role in the prevention of chill haze, and subsequent risk to permanent haze formation in fruit-based beverages or cereal based beverages, such as beer. Chill haze forms as a result of the interaction of haze-active proteins with polyphenols. In order to maintain the clarity of beer and to extend colloidal shelf life, the brewing industry selectively removes these proteins from beer by adsorption onto and into amorphous silicas. Information regarding the detailed structure and amino acid sequence of these haze-active proteins is limited. In general terms, there are two types of protein present in beer: hydrophilic and hydrophobic. The hydrophilic proteins are responsible for haze formation and need to be removed whereas the hydrophobic proteins provide foam stability and must remain<sup>16</sup>. Bentonite removes both haze-active and foam-active protein whereas silica gel offers specificity for haze-active proteins<sup>20</sup>. This specificity is a result of the proline residues in the haze-active proteins. These proline rich proteins are known to originate from malt and have an isoelectric point in the region of beer pH (pH 4.4). It is thought that the silanol OH groups on the silica may attach to proline in a manner similar to that of phenol OH groups<sup>20</sup>. The protein is then removed together with the adsorbent either by filtration or centrifuge.

A number of studies have used fluorescence, circular dichroism<sup>4</sup> and in situ ellipsometry<sup>17,19</sup> to examine protein interaction with various membranes and silica surfaces but few, if any, have looked at this area with respect to beer stabilisation.

Changes in the fluorescence lifetimes and rotational correlation times are often indicative of changes in protein conformation<sup>18</sup> and many workers have used time and frequency domain fluorescence to record lifetimes, anisotropy and fluorescence resonance energy transfer, alongside steady-state techniques to track protein dynamics<sup>3,10,11</sup>. The study of haze-active protein was masked by the intrinsic fluorescence properties of beer and for this reason, the present study used isolated haze-active protein to examine its interaction with silica gel. For this study, the intrinsic amino acid fluorescence in the isolated protein was used in preference to extrinsic probes<sup>2</sup>.

This paper discusses the application of fluorescence to monitor beer protein uptake with a view to preventing chill haze formation. The work was carried out in order to identify the optimum conditions for protein uptake by silica and perhaps elucidate the protein's conformation in solution and at the silica surface. The study involves evalu-

<sup>1</sup>Photophysics Group, Department of Physics and Applied Physics, University of Strathclyde, Glasgow, G4 0NG, UK.

<sup>2</sup>International Centre for Brewing and Distilling, Heriot-Watt University, Riccarton, Edinburgh, EH14 4AS, UK.

<sup>3</sup>INEOS Silicas Ltd., Liverpool Road, Warrington, WA5 1AB, UK.

<sup>4</sup>Corresponding author: E-mail: djs.birch@strath.ac.uk

ation of the interaction of haze-active proteins, alongside standard proteins such as BSA and HSA, with various silica structures. The process used for the isolation of haze protein is reported<sup>15</sup>, as is the use of time-resolved fluorescence anisotropy to estimate the radii of the proteins. The intrinsic fluorescence characteristics of beer are also presented and comparisons made with isolated beer constituents.

## THEORY

Fluorescence decay curves were fitted using non-linear least squares reconvolution to impulse response functions in the form of a sum of exponentials i.e.

$$I(t) = \sum_i A_i \exp\left(-t/\tau_i\right) \quad (\text{eq. 1})$$

where  $I(t)$  is the fluorescence intensity at time  $t$ ,  $\tau_i$  is the fluorescence lifetime and  $i=1,2,3\dots$  etc., as required to minimise a normalised  $\chi^2$  goodness of fit criterion<sup>5</sup>.

The theory and application of time-resolved fluorescence anisotropy has been reviewed many times for bio and organic polymers<sup>14,23</sup>. We summarise the relevant parts here.

By recording vertically and horizontally polarised fluorescence decay curves,  $F_V(t)$  and  $F_H(t)$ , orthogonal to vertically polarised excitation, an anisotropy function  $R(t)$  can be generated i.e.

$$R(t) = \frac{F_V(t) - F_H(t)}{F_V(t) + 2F_H(t)} \quad (\text{eq. 2})$$

For an unbound spherical rigid rotor in an isotropic solvent the decay of  $R(t)$ , which describes depolarisation of the fluorescence due to Brownian rotation, can be expressed as:

$$R(t) = R_0 \exp\left(-t/\tau_r\right) \quad (\text{eq. 3})$$

where  $R_0$  is the initial anisotropy with a maximum value of 0.4 and, in the simplest case,  $\tau_r$  is the rotational time of the molecule and can be described by the Stokes-Einstein equation:

$$\tau_r = \frac{\eta V}{kT} \quad (\text{eq. 4})$$

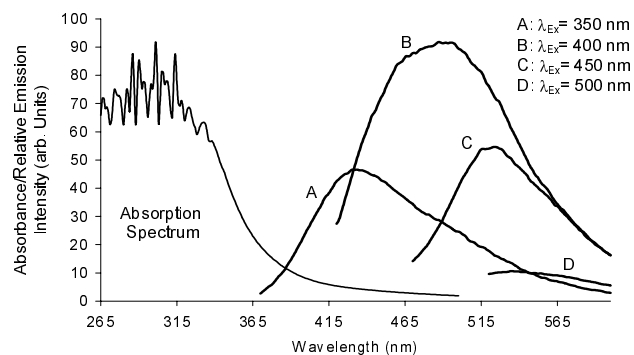


FIG. 1. Absorption and emission spectra (various  $\lambda_{\text{ex}}$ ) of undiluted lager beer.

where  $\eta$  is the microviscosity,  $V$  the hydrodynamic molecular volume,  $T$  the absolute temperature and  $k$  the Boltzmann constant.

In the case of hindered molecular rotation, the fluorescence is only partially depolarised leading to a residual anisotropy  $R_\infty$  given by:

$$R(t) = (R_0 - R_\infty) \exp\left(-t/\tau_r\right) + R_\infty \quad (\text{eq. 5})$$

In this instance  $\tau_r$  reflects the vicinal viscosity close to the binding site, which may be markedly different from that in bulk solution described by equation 3.

Protein anisotropy functions are often bi-exponential with a fast component attributed to the segmental motion of tryptophan residues within the protein and a slow component related to the overall rotational diffusion of the protein. In the case of a bi-exponential decay the anisotropy function can be expressed as:

$$R(t) = B_1 \exp\left(-t/\tau_{r1}\right) + B_2 \exp\left(-t/\tau_{r2}\right) \quad (\text{eq. 6})$$

where  $B_1$  and  $B_2$  are the relative amplitudes and  $\tau_{r1}$  and  $\tau_{r2}$  are the rotational correlation times for the various components.

We have found that equation 6 provides the best description of haze protein rotating in water, with equation 4 providing an estimate of the hydrodynamic radius of the protein.

## MATERIALS AND METHODS

### Instrumentation

The absorption spectra were recorded on a Perkin Elmer UV-vis Lambda 2 spectrometer and steady-state fluorescence emission spectra on a Perkin Elmer LS50B luminescence spectrometer. Time-resolved fluorescence measurements were made using the time-correlated single-photon counting technique<sup>5</sup>. A hydrogen filled coaxial flashlamp<sup>6</sup> with an instrumental response of 1.6 ns *fwhm* was used to study the intrinsic protein fluorescence. Wavelength selection was made using a monochromator with a 20 nm bandpass. Unless otherwise stated, excitation was centred at 280 nm. A 320 nm cut-off filter selected the fluorescence, which was detected using a Philips XP2020Q photomultiplier. To correct for intensity drifts during anisotropy measurements, Glan-Thompson polarisers were automatically toggled between the two polarisations. To study fluorescamine labelled proteins, the flashlamp was replaced by a 370 nm, 1.5 ns instrumental *fwhm*, NanoLED supplied by IBH. In this instance, a 455 nm cut-off filter selected the fluorescence. Non-linear least

TABLE 1. Silica characteristics.

Silica	A		C (activated)		D (activated)	
	Large	Small	Large	Small	Large	Small
Particle size ( $\mu\text{m}$ )	11.6	11.5	11.4	11.7		
Surface area ( $\text{m}^2/\text{g}$ )	304	723	361	683		
Pore vol. ( $\text{cm}^3/\text{g}$ )	1.8	1.3	2.0	1.1		
Pore diam. ( $\text{\AA}$ )	237	72	222	64		

squares impulse reconvolution analysis of the fluorescence data was performed using the IBH software library with a  $\chi^2$  goodness of fit criterion.

### Isolation of haze protein

Maize adjunct beer was obtained from the International Centre for Brewing and Distilling (ICBD) and silica was obtained from INEOS Silicas Ltd. All treatments were carried out at 4°C.

Silica (0.1 g) was added to 200 cm<sup>3</sup> of degassed beer and incubated for 10 min using a Gallenkamp orbital shaker. The mixture was then centrifuged for 5 min at 13000 rpm using a Sorvall RC24. The liquid was discarded and the remaining silica pellet exposed to a further 200 cm<sup>3</sup> of beer. The above process was repeated until the original 0.1 g of silica had been exposed to 4 litres of beer. At this stage, 5 cm<sup>3</sup> of 0.1 M NaOH was added to the silica pellet and the resulting mixture centrifuged for 10 min at 13000 rpm. This process was repeated and the recovered liquid was made up to 100 cm<sup>3</sup> with millipore ultra-pure deionised H<sub>2</sub>O. Ammonium sulphate (51 g) was added and the solution stirred for 30 min. The sample was then centrifuged for 10 min at 7480 rpm. The remaining pellet was added to 40 cm<sup>3</sup> of deionised water and shaken until the isolated protein was resuspended in solution. The protein was purified by dialysis and recovered by freeze-drying.

### Sample preparation

All chemicals were used as received from Aldrich Chemical Co., and were prepared using doubly distilled H<sub>2</sub>O. The pH solutions were prepared from dilute hydrochloric acid (acidic) and dilute sodium hydroxide (alkaline). All samples were cast into 1x1x4 cm cuvettes (Hughes and Hughes Ltd.).

Solutions of 5x10<sup>-6</sup> M HSA and BSA were prepared at pH 2, pH 4, pH 6, pH 8 and pH 10. Stock solutions of haze protein were prepared (10 mg protein per 100 cm<sup>3</sup> H<sub>2</sub>O), diluted to the required concentration and the pH adjusted accordingly. The isolated protein was prepared in 4% ethanol solutions in an attempt to mimic its natural alcoholic environment. Silica was added at a level of 100 g/hL. Table I details the characteristics of the silicas used, where silica C is the heat-treated version of silica A and silica D the heat-treated version of silica B. The heat treatment aims to activate the surface by maximising the number of isolated silanol groups (cf. US Patent 4515821). All solutions were in overnight contact with silica. Preliminary experiments indicated that low temperatures were required for the successful take-up of protein and so filtration took place at T < 4°C, using sintered glass funnels.

## RESULTS AND DISCUSSION

### Fluorescence characteristics of beer

Before isolating the haze protein, initial measurements were carried out on the intrinsic fluorescence of various types of beer. Fig. 1 shows the absorption and emission spectra of undiluted Tennents lager. No intrinsic protein fluorescence is evident although several emission peaks are observed at longer wavelengths (400 – 550 nm). Upon

dilution with distilled water, the intensity of the long wavelength peaks diminished and protein fluorescence, thought to originate from tryptophan/tyrosine residues present in the beer proteins, was observed. The peak at 315 nm was attributed to tyrosine and not catechin or epicatechin. The latter are removed by PVPP and yet the 315 nm peak was found to remain after PVPP treatment. Fig. 2 illustrates the fluorescence spectra of Tennents lager for a dilution of 1 drop of beer:3 cm<sup>3</sup> H<sub>2</sub>O. The fluorescence spectra shown here are typical of all beers. The same features were seen for 'All Malt', 'Maize Adjunct', 'Tennents', 'Heineken' and even 'Whitbread Best Bitter', which is an 'ale' as opposed to a 'lager' beer.

To identify the source of the intrinsic fluorescence at excitation wavelengths >300 nm, comparisons were made with various types of hops, a rich source of iso- $\alpha$ -acids which are known to impart a unique bitterness and aroma to beer<sup>9,13</sup>. The steady-state fluorescence spectra for 'Target' hops, which have been pre-isomerised and are thus already bitter, are shown in Fig. 3 and the corresponding spectra for 'Hallertau' hops, which are added towards the end of the boil to give aroma rather than bitterness, are shown in Fig. 4. Further comparisons were made with various polyphenol sources such as the proanthocyanidin monomers – catechin and epicatechin. The fluorescence characteristics of tea, a source of complex polyphenols, were also studied since polymerised polyphenols are known to be present in beer and indeed are more haze active than the simple monomer units<sup>21</sup>. The fluorescence

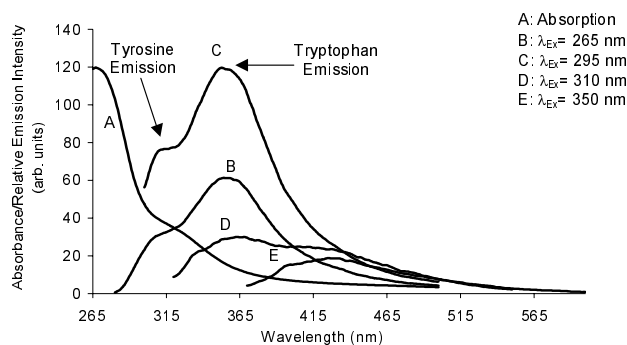


FIG. 2. Absorption and emission spectra (various  $\lambda_{EX}$ ) of diluted lager beer.

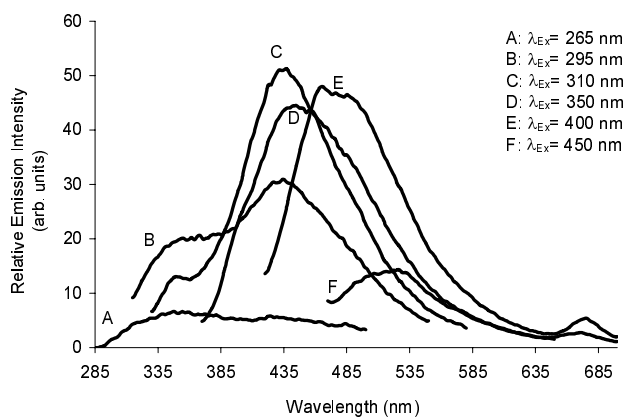


FIG. 3. Fluorescence spectra of Target hops in aqueous solution (various  $\lambda_{EX}$ ).

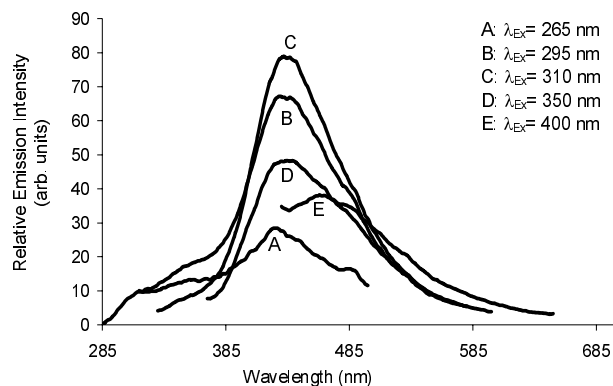


FIG. 4. Fluorescence spectra of Hallertau hops in aqueous solution (various  $\lambda_{Ex}$ ).

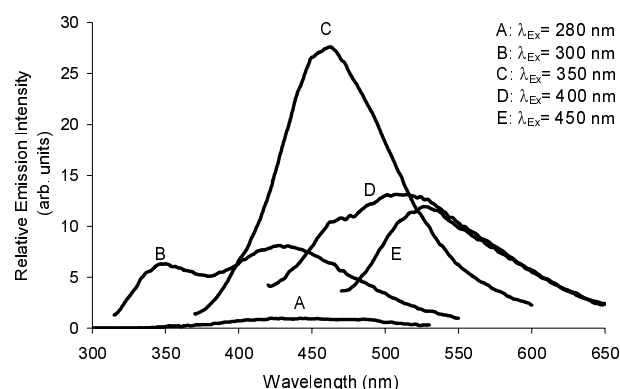


FIG. 5. Fluorescence spectra of tea (various  $\lambda_{Ex}$ ).

maximum for both catechin and epicatechin occurs at 315 nm when excited at 265 nm and 280 nm and may overlap tyrosine/tryptophan fluorescence; minimal fluorescence being observed if the excitation wavelength is increased. In contrast, the fluorescence characteristics of tea are more complex with the emission maxima occurring at longer wavelengths and changing with excitation wavelength (Fig. 5). Comparing the fluorescence spectra of beer with that of tea and hops, it seems likely that complex polyphenols, proteins and iso- $\alpha$ -acids contribute to the intrinsic fluorescence of beer although the contribution from polyphenols must be minimal since their removal by PVPP does not greatly affect the observed fluorescence.

Studying the fluorescence spectra of beer as a function of dilution, a gradual decrease was observed in the intensity of the long wavelength peaks, coinciding with a gradual increase in intensity of the protein peaks. One possible explanation for the change in spectra is that resonance energy transfer takes place between proteins (tryptophan residues) and iso- $\alpha$ -acids/polyphenols, however time-resolved fluorescence lifetime measurements suggest that this is not the case. An alternative explanation for the observed spectral change is that inner filter effects take place within the sample. To test this, the optical density was measured for tryptophan in aqueous solution. When compared with the results for beer, the weakest sample (1 drop of beer:3 cm<sup>3</sup> H<sub>2</sub>O) had an optical density of ~ 0.55. As soon as the concentration of the beer reached 0.1 cm<sup>3</sup> beer:2.90 cm<sup>3</sup> of H<sub>2</sub>O, the optical density rose to 1.38 indicating that inner filter effects were responsible for the observed changes.

As has been illustrated, the fluorescence characteristics of beer are far from simple. To further characterise this

Table II. Fluorescence decay components of diluted Tennents lager before and after treatment with silica, tannic acid and polyvinylpyrrolidone ( $\lambda_{Ex}$ =280 nm). The % contribution of each component to the total intensity and the standard deviations are also shown.

Filename	1-exp. $\chi^2$	2-exp. $\chi^2$	3-exponential fit			$\chi^2$
			$\tau_1/s$ (Std. dev.)	$\tau_2/s$ (Std. dev.)	$\tau_3/s$ (Std. dev.)	
Untreated Tennents	59.10	5.670	2.76E-10 (3.40E-11) 21.61%	2.86E-9 (3.43E-11) 52.65%	1.11E-8 (9.90E-11) 25.74%	1.204
Silica treated	55.70	6.477	3.06E-10 (3.48E-11) 22.50%	3.00E-9 (3.52E-11) 55.18%	1.19E-8 (1.23E-10) 22.32%	1.390
PVPP treated	55.33	5.856	3.27E-10 (4.03E-11) 21.47%	2.96E-9 (3.57E-11) 55.20%	1.16E-8 (1.16E-10) 23.33%	1.378
Tannic acid treated	53.91	6.267	2.71E-10 (5.10E-11) 22.53%	2.97E-9 (3.30E-11) 55.23%	1.19E-8 (1.16E-10) 22.24%	1.400

Table III. Steady-state fluorescence results for the interaction of Tennents lager with silica, tannic acid and polyvinylpyrrolidone (various dilutions, various  $\lambda_{Ex}$ ).

$\lambda_{Ex}$ (nm)	Dilution (beer : H <sub>2</sub> O) (cm <sup>3</sup> )	Fluorescence intensity (arb. units)				Fluorescence $\lambda_{max}$ (nm)
		Untreated	Silica treated	PVPP treated	Tannic acid treated	
280	Undiluted	-	-	-	-	-
	1.50 : 1.50	-	-	-	-	-
	0.25 : 2.75	43	52	52	57	365
	1 drop : 3.00	99	81	107	109	355
350	Undiluted	8	7	8	9	445
	1.50 : 1.50	50	58	59	63	435
	0.25 : 2.75	125	132	131	129	430
	1 drop : 3.00	35	24	36	36	430
450	Undiluted	84	86	88	82	520
	1.50 : 1.50	65	69	68	62	517
	0.25 : 2.75	17	17	17	15	515
	1 drop : 3.00	3	2	3	3	525

intrinsic fluorescence, time-resolved fluorescence lifetime measurements were performed on both diluted and undiluted beer samples and a typical decay curve is shown in Fig. 6 (diluted Tennents, 1 drop of beer:3 cm<sup>3</sup> H<sub>2</sub>O). Lifetime measurements were also performed before and after the removal of haze-active proteins (treatment with silica and tannic acid) and polyphenols (treatment with polyvinylpyrrolidone, PVPP). The measurements failed to show the uptake of the relevant constituents. The fluorescence lifetimes obtained from these measurements are tabulated in Table II for a dilution of 0.5 cm<sup>3</sup> beer:2.5 cm<sup>3</sup> H<sub>2</sub>O; further dilution of the beer before the respective treatments did not alter any of the parameters. The corresponding steady-state results for various dilutions of beer are shown in Table III; again, no change was observed.

Failure to change the fluorescence characteristics of beer by exposing it to silica, PVPP or tannic acid led to a study of the isolated haze-active protein. Isolation of this component allows the interaction with silica to be moni-

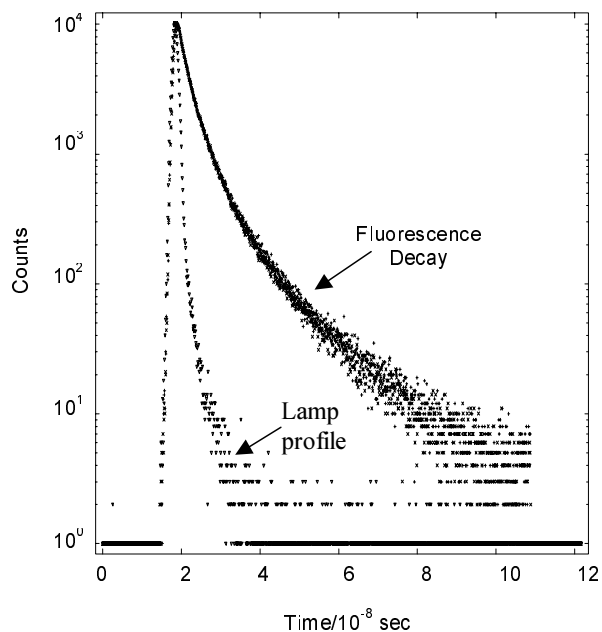


FIG. 6. Time-resolved fluorescence decay of diluted Tennents lager ( $\lambda_{EX}=280$  nm).

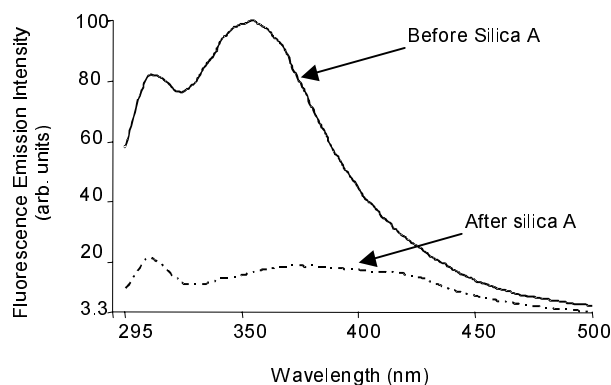


FIG. 7. Fluorescence spectra of isolated haze protein before and after silica treatment ( $\lambda_{EX}=280$  nm).

tored in a controlled and simple environment rather than against the background of beer fluorescence.

### Silica interaction with isolated haze protein

The steady-state fluorescence spectra of isolated haze protein (pH 4, 4% (w/v) ethanol solution), before and after silica treatment, are shown in Fig. 7 and the pH dependence of this uptake is illustrated in Table IV, where the % reduction is defined by equation (7),

$$\%Reduction = \frac{Intensity\ decrease}{Original\ intensity} \times 100\% \quad (eq. 7)$$

where  $Intensity\ decrease = (original\ intensity - final\ intensity)$ .

In contrast to the studies on beer (Tables II and III), Fig. 7 clearly shows protein uptake. The observed de-

Table IV. Steady-state fluorescence results for isolated haze protein uptake as a function of pH ( $\lambda_{EX}=280$  nm).

pH	Relative Emission Intensity (arb. units)		
	Before Silica A	After Silica A	% Reduction
2	85	15	82
4	100	17	83
6	101	27	73
8	97	27	72
10	100	35	65

Table V. Steady-state fluorescence results for the interaction of haze protein, pH 4, with silicas A – D ( $\lambda_{EX}=280$  nm, fluorescence  $\lambda_{max}=355$  nm).

Silica	Relative Emission Intensity (arb. units)		
	Before Silica	After Silica	% Reduction
A	94	16	83
B	99	16	84
C	100	16	84
D	98	25	75

Table VI. Time-resolved anisotropy results using fluorescamine as an extrinsic fluorescence probe.

Rotational time(s)	Protein	$\tau_r$ (ns)	Radius			$\chi^2$
			$\tau_{r1}$ (ns)	$\tau_{r2}$ (ns)	(nm)	
One	HSA	41.7				1.908
	BSA	12.5				2.792
	Haze	10.2				1.646
Two	HSA	3.30	78.0	4.22		1.101
	BSA	2.40	56.8	3.80		1.176
	Haze	1.56	48.1	3.59		1.142

Table VII. Time-resolved anisotropy results using the intrinsic protein fluorescence.

Rotational time(s)	Protein	$\tau_r$ (ns)	Radius			$\chi^2$
			$\tau_{r1}$ (ns)	$\tau_{r2}$ (ns)	(nm)	
One	HSA	43.6				1.101
	BSA	32.7				1.064
	Haze	2.22				1.289
Two	HSA	0.48	56.1	3.78		1.007
	BSA	0.69	36.4	3.27		1.049
	Haze	0.66	37.5	3.31		1.038

crease in fluorescence intensity can be directly related to the removal of protein from solution i.e. protein is adsorbed by the silica and subsequently removed during the filtration process. Initial measurements indicate that for haze-active protein the maximum uptake occurs at pH 2 – pH 4 (Table IV), which is convenient for the brewing industry as pH 4.4 is the typical pH of beer.

Fig. 7 clearly shows a spectral shape change after silica treatment. One possible explanation for the change is that a protein-polyphenol complex was present prior to the isolation process. This could result in isolation of haze protein with a small proportion of polyphenol attached, resulting in some long wavelength fluorescence. Time-resolved fluorescence lifetime measurements confirm the presence of a residual impurity, with apparent variations in the lifetimes before and after silica treatment<sup>1</sup>. A typical fluorescence decay of aqueous haze protein, pH 4, is illustrated in Fig. 8; the fitted function and weighted residuals are also shown. The fluorescence lifetime is best described by a triple exponential function and gives a  $\chi^2$  of 1.12 with lifetimes of 0.26 ns (22.17%), 2.76 ns (57.17%) and 6.21 ns (20.66%). The fluorescence decay is triple exponential regardless of pH although the magnitude and relative percentages do vary.

Working only at the pH of interest to the breweries i.e. pH 4, fresh samples were prepared and exposed to silicas A – D. As before, samples were in overnight contact with the silica and filtered by gravity using sintered glass funnels. The results shown in Table V indicate that silica D, an activated, small pore size, large surface area silica was the least effective at removing haze protein. Very little difference was observed in the performance of the other silicas.

### Silica interactions with standard protein solutions

Similar experiments were performed using the standard, well-characterised proteins HSA and BSA to identify optimum conditions for uptake. Steady-state and time-resolved fluorescence measurements were carried out on

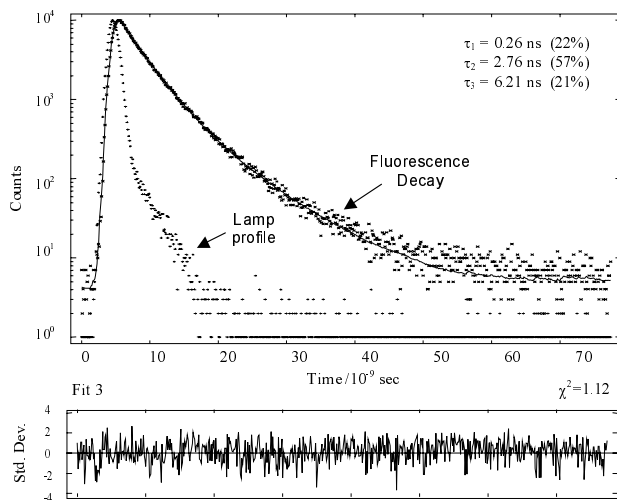


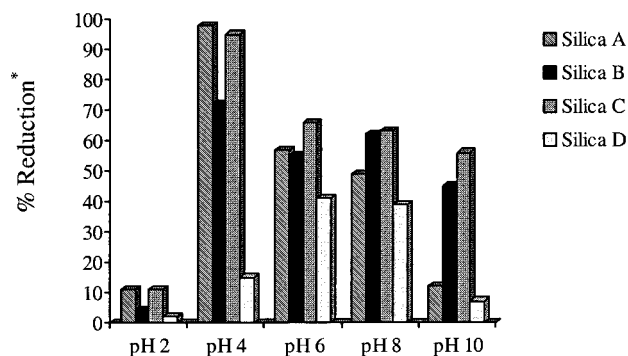
FIG. 8. Time-resolved fluorescence decay, fitted function and weighted residuals for an aqueous solution of isolated haze protein, pH 4,  $\lambda_{\text{Ex}} = 280 \text{ nm}$ .

the protein solutions before and after treatment with silicas A - D. Interactions were monitored over the range pH 2 – pH 10. The corresponding steady state results for HSA and BSA are illustrated in Figs. 9 and 10.

As shown in Figs. 9 and 10, silicas A and C were more efficient than B and D. Comparison with Table I indicates that the most efficient silicas for the uptake of BSA and HSA are those with large pore diameters but relatively small surface areas. This is not surprising since both HSA and BSA are large globular proteins (radius  $\sim 4 \text{ nm}$ ). The favoured silicas suggest that these proteins are buried in the pores of the silica and are not surface bound. The efficiency of take-up varies quite considerably with pH, pH 4 being optimum. This concurs with the findings for haze-active protein. The strong pH dependence indicates the involvement of H-bonding and other electrostatic interactions. It should be noted that although pH 4 was found to be optimum for the proteins discussed here, it does not apply to every protein and was in fact found to be the least effective pH for uptake of Subtilisin Carlsberg<sup>1</sup>, another widely studied single tryptophan protein.

### Time-resolved fluorescence anisotropy

Time-resolved fluorescence anisotropy measurements were performed to determine the protein hydrodynamic radii using both intrinsic and extrinsic fluorophores. Fluor-



$$* \% \text{ Reduction} = \frac{\text{Intensity decrease}}{\text{Original intensity}} \times 100\%$$

FIG. 9. Percent reduction in fluorescence intensity of HSA after silica treatment.

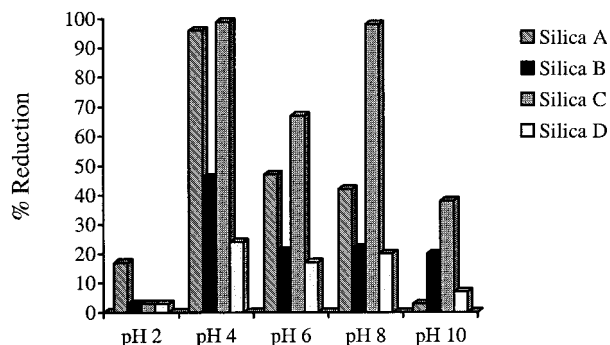


FIG. 10. Percent reduction in fluorescence intensity of BSA after silica treatment.

rescamine was chosen as the extrinsic probe because of its effective binding to amino acids, ease of sample preparation and the fact that any free fluorescamine present is non-fluorescent in H<sub>2</sub>O<sup>7,8,22</sup>; its suitability for use with the NanoLED ( $\lambda_{\text{ex}} = 370 \text{ nm}$ ) facilitates reduced acquisition times and more accurate data collection. To check on the suitability of fluorescamine for this type of study, the standard proteins HSA and BSA were also labelled, and the anisotropy results compared with those obtained using the H<sub>2</sub> flashlamp to excite the intrinsic amino acid fluorescence of the proteins. Various models were used to analyse the data, but the two rotational times model (equation 6) was found to be most appropriate. The results for both one and two rotational time models are shown for comparison. Table VI details the results obtained for HSA, BSA and haze protein using the extrinsic probe, and Table VII provides the corresponding results obtained via the intrinsic fluorescence. The protein radii were calculated using  $\tau_2$  in equation 4 with a viscosity of 1 cP.

Comparing both sets of results, the most noticeable difference occurs in the magnitude of  $\tau_1$ . Exciting the intrinsic fluorophore results in a fast correlation time that is a fraction of a nanosecond whereas with the extrinsic probe, the fast component is somewhat slower reflecting the relative size and mobility of the fluorophores. The calculated protein radii are slightly larger when the extrinsic probe is used. This might be expected as no allowance was made for the size of the probe molecules attached to the protein and indeed energy transfer between amino acid fluorophores might cause a more rapid depolarisation. The intrinsic and extrinsic results are relatively consistent and both indicate the haze protein has an approximate radius of 35 Å. The radii calculated for HSA and BSA are in good agreement with the accepted values of 40 Å and 35 Å respectively<sup>12</sup>.

## CONCLUSION

The fluorescence characteristics of beer have been explored with the identification of complex polyphenols and iso- $\alpha$ -acids as possible sources of intrinsic beer fluorescence. The uptake of protein by silica was monitored via steady-state and time-resolved fluorescence techniques, with particular attention being paid to those fractions involved in chill haze formation. Large pore volume, small surface area silicas were found to be the most efficient adsorbers of BSA and HSA, whereas the non-activated small pore volume silica (silica B) was one of the most efficient adsorbers of isolated haze protein. In all instances the performance of silica D, the activated version of silica B, was very poor. One of the main factors that affect the extent of the adsorption is pH; this indicates that H-bonding and other electrostatic interactions are involved in the adsorption process. Time-resolved fluorescence anisotropy gave an estimated radius of 35 Å for haze protein. The use of fluorescamine as a protein label has been demonstrated

and its properties may prove useful in determining protein content in beer. Foam-forming proteins have also been isolated and similar studies to those given here, alongside a comparison with isolated haze protein, will be reported shortly.

## ACKNOWLEDGEMENTS

We wish to acknowledge the EPSRC and INEOS Silicas Ltd. for financial support, and Tennents Caledonian Brewery for supplying additional beer. We also thank Prof. G. Krishnamoorthy for helpful suggestions.

## REFERENCES

1. Apperson, K., PhD Thesis, 2002, University of Strathclyde.
2. Bamforth, C. W., Kapp, G. R. and Smythe, J. E., *Food Chemistry*, 2001, **73**, 377.
3. Beecham, J. M. and Brand, L., *Annual Review of Biochemistry*, 1985, **54**, 43.
4. Billsten, P., Wahlgren, M., Arnebrant, T., McQuire, J. and Elwing, H., *Journal of Colloid and Interface Science*, 1995, **175**, 77.
5. Birch, D. J. S. and Imhof, R. E., In: Topics in Fluorescence Spectroscopy, Volume 1: Techniques, J. R. Lakowicz, Ed., Plenum Press: New York, 1991, pp.1-95.
6. Birch, D. J. S. and Imhof, R. E., *Review of Scientific Instruments*, 1981, **52**, 1206.
7. Castell, J. V., Cervera, M. and Marco, R., *Analytical Biochemistry*, 1979, **99**, 379.
8. De Bernardo, S., Weigele, M., Toome, V., Manhart, K., Leimgruber, W., Bohlen, P., Stein, S. and Udenfriend, S., *Archives of Biochemistry and Biophysics*, 1974, **163**, 390.
9. De Keukeleire, D., *Quimica Nova*, 2000, **23**, 108.
10. Eftink, M. R., In: Methods of Biochemical Analysis, C. H. Suelter, Ed., John Wiley and Sons: New York, 1991, pp.128-205.
11. Eftink, M. R., *Biophysical Journal*, 1994, **66**, 482.
12. He, X. M. and Carter, D. C., *Nature*, 1992, **358**, 209.
13. Hughes, P., *Journal of the Institute of Brewing*, 2000, **106**, 271.
14. Lakowicz, J. R., Principles of Fluorescence Spectroscopy, Kluwer Academic/Plenum: New York, 1999.
15. Leiper, K., PhD Thesis (data to be published), 2002, Heriot-Watt University.
16. Lewis, M. J. and Young, T. W., *Brewing*, Chapman and Hall: London, 1995.
17. Malmsten, M. and Veide, A., *Journal of Colloid and Interface Science*, 1996, **178**, 160.
18. Maste, M. C. L., Norde, W. and Visser, A. J. W. G., *Journal of Colloid and Interface Science*, 1997, **196**, 224.
19. McQuire, J., Singla, B. and Krisdhasima, V., *Journal of Colloid and Interface Science*, 1996, **182**, 292.
20. Siebert, K. J. and Lynn, P. Y., *Journal of the American Society of Brewing Chemists*, 1997, **55**, 73.
21. Siebert, K. J. and Lynn, P. Y., *Journal of the American Society of Brewing Chemists*, 1998, **56**, 24.
22. Stein, S., Bohlen, P., Stone, J., Dairman, W. and Udenfriend, S., *Archives of Biochemistry and Biophysics*, 1973, **155**, 202.
23. Steiner, R. F., In: Topics in Fluorescence Spectroscopy, Volume 2: Principles, J. R. Lakowicz, Ed., Plenum Press: New York, 1991, pp.1-52.

(Manuscript accepted for publication April 2002)