

A Method of Detection for Residual Isinglass in Filtered and Cask-Conditioned Beers

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ABSTRACT

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Isinglass has been used as a clarification agent in brewing for many years. However, recently its use has come under scrutiny. Due to concerns about food intolerance and food allergies, the EU and other government bodies have introduced legislation obliging food producers to declare those ingredients, additives and process aids, which are listed as allergens, on product labels. One of the affected products that is used in the brewing industry is isinglass. Within the EU, the brewing industry has objected to its inclusion as there is no evidence to suggest it is involved in food allergies. Due to this objection, isinglass has been granted exemption from the regulations for two years, to November 2007. During this time the industry must prove that isinglass is non-allergenic. This paper presents a simple and sensitive method for detecting the presence of isinglass in beer. The method involves hydrolysing beer samples with acid and measuring levels of hydroxyproline. This amino acid is found in isinglass but not in brewing raw materials. This method will be of use in any investigations involving isinglass. Data are presented showing how the method can be used to test for the presence or absence of isinglass in filtered and cask-conditioned beers.

Key words: Cask-conditioning, filtration, hydroxyproline, isinglass.

INTRODUCTION

Isinglass has been used in the brewing industry as a clarification agent for many years without attracting attention. However, in recent years this has changed. Due to increased concerns about food intolerance and allergenicity, several countries have decided that the consumer should have more information about the substances present in foods and beverages. This involves listing potential allergens present in ingredients and process aids on food labels.

In the EC, this area is covered by Directive 2000/13/EC amended by Directive 2003/89/EC. These directives enforced allergen labelling for food and drink from the 25th of November 2005. Of the categories relevant to the brewing industry, one is “Fish and Products Thereof” which includes isinglass².

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Brewers consider this to be inappropriate, as isinglass has been in use for many years without any intolerance problems being reported. Exemptions from the list of potential allergens are allowed and following representations from industry bodies, isinglass has been granted a temporary exemption for two years². It is now the responsibility of the industry to prove that isinglass is not allergenic so that a permanent exemption can be obtained.

Other countries have introduced similar legislation. In the United States, potential food allergens, including isinglass, must be listed on labels on packaged foods from the 1st of January, 2006, and allergen regulations for alcoholic beverages are under discussion. Such labelling has been required in Australia and New Zealand since December 2000 and in Japan since April 2002. Canada is considering an exemption for fining agents from its labelling regulations².

The brewing industry, together with the producers of isinglass, is now investigating the potential allergenicity of isinglass. Of use to this process would be a rapid, accurate and sensitive method for the detection of isinglass in packaged and cask-conditioned beers.

Isinglass is a pure form of collagen and is obtained by processing the dried swim bladders of certain tropical fish. Collagen contains a high proportion of the amino acids proline and hydroxyproline resulting in a high hydroxyl content. This causes a high hydrogen bonding potential which gives isinglass its fining ability. The solubilised isinglass molecule is *amphoteric* meaning that it possesses both positive and negative charged areas and can thus bind to both negatively charged yeast cells and positively charged proteins.

Fining accelerates particle settling by increasing their size and weight. Isinglass treated beers have been shown to exhibit improved foam stability, possibly due to the removal of lipid material^{4,6}. Isinglass is removed at filtration and is not present in packaged beer, although it remains in cask-conditioned beer.

This paper presents a detection method for isinglass, where HPLC is used to detect the presence of the amino acid hydroxyproline, following acid hydrolysis of beer samples. This unusual amino acid is common in collagen, but is not found in cereals or hops. Consequently if hydroxyproline is found in beer it is most likely to have originated from isinglass. The method described in this paper is highly sensitive and can detect amino acids at concentrations as low as 3pM (3.9×10^{-13} mg/L). Results from two experiments are presented showing how this method can be used to detect for the presence or absence of isinglass in filtered and cask-conditioned beers.

MATERIALS AND METHODS

Beer production

A sales gravity all malt ale was produced in the ICBD's two-hectolitre pilot brewery. The wort had an original gravity of 1040 (10° Plato) and was fermented using an industrial ale yeast. An ale was chosen as isinglass is generally used for clarification of this type of beer. Following primary fermentation and a short period of conditioning, the brew was divided, 5 L was retained as an untreated/unfiltered sample, 10 L was retained for treatment and filtration, and the remainder was placed in casks.

Treatment and filtration

The beer samples were treated with Vicfine FFV161, a powdered form of isinglass, a gift from AB Vickers (Burton-on-Trent, Staffordshire, England). The powder was dissolved in water to give a stock solution of 5000 ppm according to the manufacturer's instructions. This solution was used to treat aliquots of beer at concentrations of 1.8, 4.5 and 7.2 g/hL. These levels cover the dosage range recommended by the manufacturer. The samples were mixed for 15 min before filtration using a small scale filtration unit (Schenck) and diatomaceous earth. An untreated sample was also filtered. All preparation, treatment and filtration was carried out at 4°C.

Stability of filtered beers

The filtered beers were analysed for haze using a Hach 2100N Turbidimeter, for tannoids and sensitive proteins using a Pfeuffer Tannometer¹, for foam stability using the method of Rudin⁵ and for hydroxyproline as described below.

Cask-conditioning

Four 40 L casks were filled with 35 L of beer. One cask was left untreated as a control. The other three were treated with isinglass during filling at concentrations of 1.8, 4.5 and 7.2 g/hL. The casks were left to condition for 7 days at 5°C. After allowing 30 min for the casks to settle, they were sampled by drawing beer through a tap attached to the opening in the end of the cask (this was approximately 5 cm from the bottom of the cask end). Further samples were taken every 12 h after filling. These samples were analysed for haze as described above, for yeast cell number using an Improved Neubauer Haemocytometer and a microscope, and for the presence of hydroxyproline as described below.

Analysis of hydroxyproline

The amino acid composition of the samples was measured by hydrolysis followed by measurement of the free amino acids by gradient elution High Performance Liquid Chromatography (HPLC) using fluorescence as a means of detection. This is an adaptation of the method described by Mackey and Beck³.

Samples were reacted with the fluorescent dye dansyl chloride. The samples were bound to an HPLC column and the individual amino acids eluted using an eluent gradient. The solution coming off the column was passed

through a continuous fluorescent detector which measured the amount of dansyl chloride, this being proportional to the amount of amino acid. Each sample included an internal standard, and the system was calibrated with a calibration standard containing known concentrations of amino acids.

To prepare the samples for HPLC analysis, they were hydrolysed to split the proteins into their constituent amino acids. The samples were subjected to a one-stage hydrolysis. Approximately 200 µL of sample was placed in a screw cap Pyrex glass tube, 800 µL of 7.5 M hydrochloric acid was added (645 mL concentrated acid diluted to 1 L with water) and the tubes sonicated for 15 min. The tubes were then placed into a heating block pre-heated to 110°C (Dri-block DB-3, Techne) and left for 24 h. This method results in the destruction of the sulphur-containing amino acids methionine and cysteine. These acids can be detected, if desired, by using a preliminary hydrolysis stage using performic acid, followed by the addition of sodium metabisulphite, but this stage was omitted for this work.

Samples were removed from the heating block and allowed to cool. The contents were diluted with water to 5 mL in volumetric flasks. Samples were filtered through 0.45 µm filters and the filtrates retained and 4 mL of each filtrate was placed in a 250 mL round bottomed flask and attached to a rotary evaporator. Samples were evaporated to dryness and re-suspended in 800 µL sodium carbonate (0.2M pH 9.7). Samples were frozen until analysed.

The samples were dansylated by placing 20 µL sample or standard in a 1.5 mL screw-capped reaction vial, 200 µL sodium carbonate (0.2 M pH 9.7), 20 µL internal standard and 200 µL dansyl chloride (5 mg in 1 mL acetone) were added. The tubes were sealed, vortexed and kept in the dark at room temperature overnight. After incubation, the volume of the samples was made up to 1 mL with water at pH 9.7. Each sample was dansylated neat and diluted 1 in 20. A standard curve was prepared by adding known amounts of hydroxyproline to beer. These samples were treated in the same way as the samples.

The internal standard solution was made up as follows. A 100 mM solution of norvaline was made in 0.1 M HCl, this was diluted 1/200 to give a 0.5 mM solution. This was frozen in aliquots. When 20 µL of this was placed in a sample, this gave 10 nM/mL, corresponding to 200 pM per 20 µL when injected.

The calibration standard was made up as follows: 100 mM solutions of cysteic acid, hydroxyproline, homoserine, taurine, ornithine, 40 mM norleucine and 50 mM methionine sulphone were prepared in 0.1 M HCl. Of this, 25 µL of the 100 mM, 62.5 µL of the 40 mM and 50 µL of the 50 mM solutions were added to 1 mL of the Sigma amino acid standard solution AA-S-18. This contained amino acids at 2.5 mM, except cysteine at 1.25 mM. The volume was made up to 5 mL with 0.1 M HCl, this produced a standard solution containing 0.5 mM of each amino acid except cysteine at 0.25 mM. This was frozen in aliquots. When 20 µL of this was dansylated and diluted to 1 mL it produced a solution containing 10 nM/mL of each amino acid except cysteine at 5 nM/mL. When 20 µL was injected, this corresponded to 200 pM of each amino acid except cysteine at 100 pM.

Table I. HPLC eluent gradient programme for amino acids.

Time (minutes)	% Eluent A	% Eluent B
0	93	7
35	87	13
45	87	13
50	80	20
60	72	28
70	64	36
80	56	44
85	52	48
95	44	56
100	40	60
105	30	70
110	30	70
110.1	93	7
145	93	7

Eluents were prepared by first preparing 20 mM di-sodium hydrogen orthophosphate, 5.678 g was dissolved in 1.9 L of water, the pH was buffered to 6.19 and the volume made up to 2 L in a volumetric flask. Eluent A consisted of the di-sodium hydrogen orthophosphate solution with 5% acetonitrile added. Eluent B consisted of a 40:60 solution of di-sodium hydrogen orthophosphate solution and acetonitrile.

The instrumentation used comprised a Gilson 231 auto-sampler with a 401 dilutor, a Rheodyne 7010 injector with a 20 µL loop, a Gilson 306 pump with a 5SC pump head, a Gilson 302 pump with a 5SC pump head, a Gilson 802 Manometric controller, a Gilson 811C dynamic mixer, a Jasco FP1520 fluorescence detector, a Gilson 715 data handling package and a Phenomenex Degassex DG4400 degassing unit. The column, a Phenosphere-NEXT 5 µm C18 150 × 4.6 mm (Phenomenex UK Ltd.), was placed in an oven at 27°C.

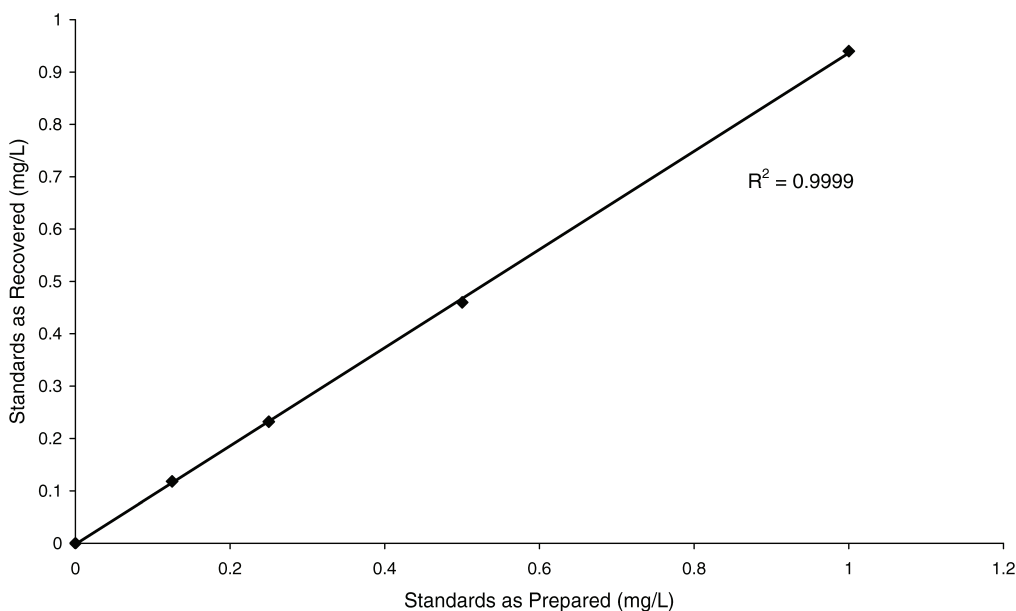
The pump programme to create the eluent gradient is shown in Table I. The increasing amount of Eluent B removed the amino acids from the column. The flow rate was 1.5 mL/minute and the back pressure 1900 psi. All

Table II. HPLC retention times of amino acids.

Amino acid	Time (min)
Cysteine as cysteic acid	21.58
Aspartic acid	22.50
Glutamine and glutamic acid	25.09
Hydroxyproline	43.83
Homoserine	52.76
Serine	53.55
Arginine	54.02
Threonine	56.71
Glycine	57.81
Alanine	59.89
Methionine as methionine sulphone	62.77
Taurine	63.67
Proline	64.12
Valine	69.07
Norvaline (internal standard)	71.55
Methionine	72.39
Isoleucine	75.33
Leucine	76.11
Norleucine	78.54
Phenylalanine	79.95
Cysteine (in free form)	85.73
Ornithine	100.20
Lysine	100.94
Histidine	101.42
Tyrosine	109.71

the amino acids gave a linear response to 250 pM injected and cysteine was linear to 300pM. All amino acids were detectable at 6–10pM injected. A run of 5 individually dansylated standards in duplicate (10 injections) gave a mean standard deviation of 2.38%, the mean standard deviation for duplicates was 1.28%. The retention times of the individual amino acids are shown in Table II.

A blank dansylation sample produced small peaks at the retention times of glutamic acid, threonine and proline. These values were subtracted from the peak values to give the true amounts of these amino acids. Results were expressed in nM/mL and were converted to give mg/L.

**Fig. 1.** Standard curve of hydroxyproline in beer.

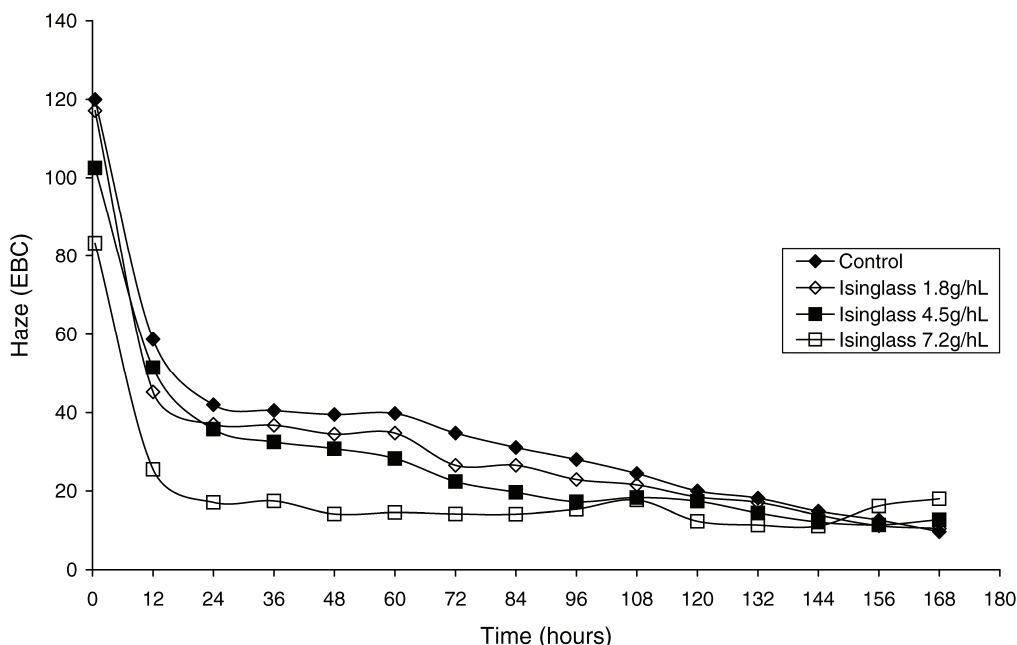


Fig. 2. Effect of isinglass levels on haze precipitation during cask-conditioning.

RESULTS AND DISCUSSION

Standard curve

The hydroxyproline standard curve produced is shown in Fig. 1. The curve is presented showing the weight of hydroxyproline added against the amounts recovered. It can be seen that a straight line was obtained and the data illustrated gave an r^2 value of 0.9999 and a recovery rate of 95%. The curve passes through the origin showing that the method is of use even when measuring small amounts of hydroxyproline. These features indicate that the method is an accurate and sensitive method for the detection of isinglass in beer.

Detection of isinglass in filtered beer

Data on the unfiltered and filtered beers are shown in Table III. The beer before filtration was very cloudy (120.0 EBC), it contained high levels of tannoids (75.8 mg/L PVP) and sensitive proteins (27.4 EBC) and had good foam stability (89.5 sec). Filtration reduced the haze to 0.38 EBC, the levels of tannoids and sensitive proteins by approximately 20% and had little effect on foam stability.

The use of isinglass had little effect on tannoid and sensitive protein concentrations, as expected. The main benefit of using isinglass at this stage of the brewing process was to remove yeast cells and large proteins in order to reduce the amount of material that has to be removed at

the filter, thus allowing longer filter runs. This benefit can be seen in the lower haze results for the treated beers. If an extended shelf-life is desired for a filtered beer, then silica gel or PVPP should be used. The use of isinglass resulted in a slight increase in foam stability (Table III).

No hydroxyproline was detected in the two untreated beer samples. This indicates that there was no hydroxyproline in beer, demonstrating that none of the brewing raw materials used contained this amino acid. The three treated beers also tested negative, indicating that filtration removed all of the isinglass used.

Detection of isinglass in cask-conditioned beer

The complete removal of isinglass from beer that was filtered prior to packaging was a feasible process, but this was not the case with cask-conditioned beer. Here, the isinglass remained in the cask as part of the sediment which formed as the cask was allowed to settle, usually in the cellar of the retail outlet. What was required here was an indication that the liquid in the cask was free of isinglass.

To investigate this, four casks were prepared as described previously and sampled at intervals during the settling process. The levels of haze, yeast cell number and hydroxyproline during settling are shown in Figs. 2–4 respectively. It can be seen that the haze level in the control cask fell rapidly up to 24 h, remained static up to 60 h, and then fell slowly thereafter. The effect of isinglass

Table III. Stability of unfiltered and filtered beers.

Sample	Haze (EBC)	Tannoids (mg/L PVP)	Sensitive proteins (EBC)	Foam stability (s)	Hydroxyproline (mg/L)
Untreated and unfiltered	120.0	75.8	27.4	89.5	Not detected
Untreated and filtered	0.38	61.4	21.8	88.8	Not detected
Treated 1.8 g/hL	0.26	60.4	21.8	91.7	Not detected
Treated 4.5 g/hL	0.26	58.8	22.2	93.0	Not detected
Treated 7.2 g/hL	0.25	58.3	22.8	90.7	Not detected

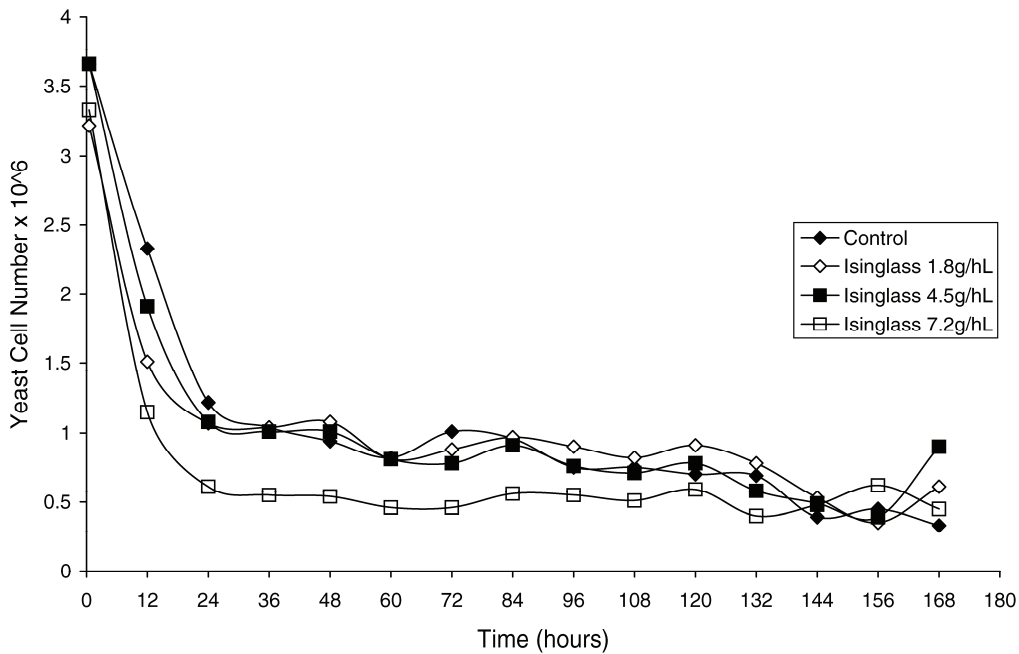


Fig. 3. Effect of isinglass levels on yeast sedimentation during cask-conditioning.

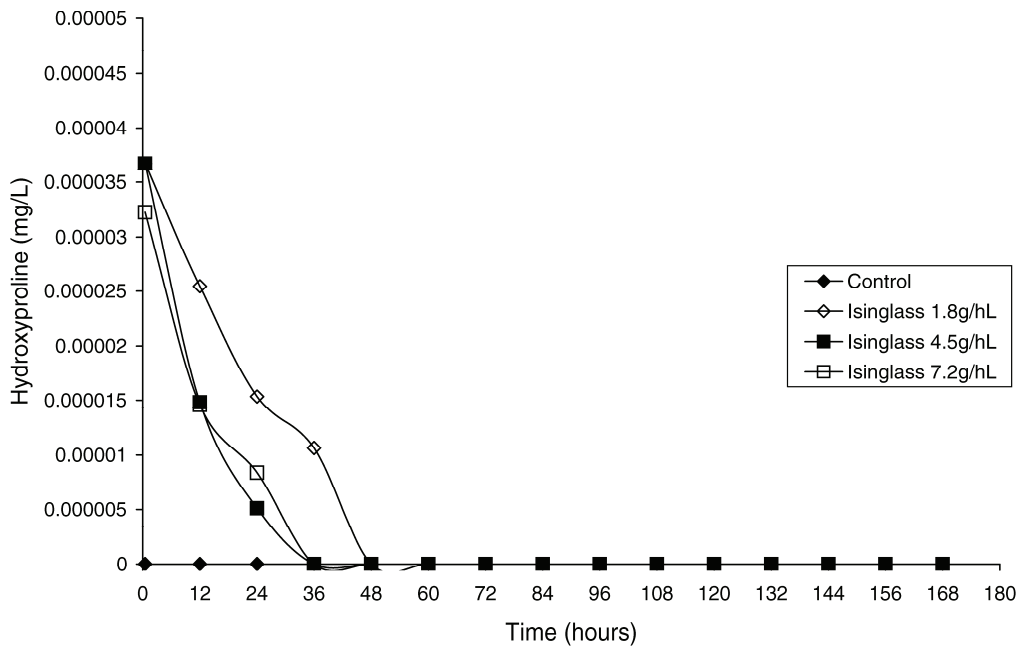


Fig. 4. Levels of hydroxyproline during cask-conditioning.

on haze removal can be seen clearly in the results from the cask treated at 7.2 g/hL where the haze level was significantly reduced after only 30 min contact (Fig. 2). Clarification was accelerated by the presence of isinglass, particularly at the 7.2 g/hL loading where the lowest haze level was achieved by 24 h. All four beers had similar clarities by 108 h of settling.

A similar pattern was observed with yeast cell number, although reduction was not observed as rapidly as with the haze results (Fig. 3). Cell number decreased rapidly during the first 24 h, and this process was accelerated by the presence of isinglass, particularly at the 7.2 g/hL loading.

The levels of hydroxyproline are shown in Fig. 4. No hydroxyproline was detected in the control as expected. In the treated casks, hydroxyproline was detected during the early stages of settling, the cask treated at 7.2 g/hL showed a lower level of hydroxyproline after 30 min settling, in agreement with the haze results. This indicates the rapidity of isinglass fining. No hydroxyproline was detected after 48 h treatment indicating that by this time the isinglass had all settled. The cask dosed at 1.8 g/hL took the longest time to settle, probably due to the lighter flocs formed because of the lower amount of isinglass used.

These experiments showed that if a cask is given time to settle properly, the beer dispensed will be free of isin-

glass. It is therefore important that the supplier's instructions to the retail outlet are observed.

CONCLUSION

The issues relating to the allergenicity of isinglass and other process aids will take time to resolve. The authors consider that this simple and sensitive method of isinglass detection using the measurement of hydroxyproline will be of use to both researchers and industry in assisting the resolution of this problem.

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REFERENCES

1. Chapon, L., Nephelometry as a method for studying the relations between polyphenols and proteins. *J. Inst. Brew.*, 1993, **99**, 49–56.
2. Long, D. and McCrimmon, E., EU regulation: The road ahead. *Brewers' Guardian*, 2005, **134**, 29–31.
3. Mackey, L.N. and Beck, T.A., Quantitative high-performance liquid chromatographic determination of sulfur amino acids in protein hydrolysates. *J. Chromatography*, 1982, **240**, 455–461.
4. Pauls Malts Brewing Room Book, 1998–2000, Pauls Malt: Ipswich, pp. 243–244.
5. Rudin, A.D., Measurement of the foam stability of beers. *J. Inst. Brew.*, 1957, **63**, 506–509.
6. Taylor, R. Traditional clarification procedures with modern cost benefits. In: Pauls Malts Brewing Room Book 1998–2000, Pauls Malt: Ipswich, pp. 78–80.

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ERRATA

A correction was made in this paper on April 13, 2006. On page 3, in the fourth paragraph of the introduction, the second sentence was revised to state that U.S. regulations requiring that potential allergens be listed on labels apply to packaged foods and that regulations for alcoholic beverages are under discussion.

A correction was made in this paper on October 16, 2006. On page 5, in the second paragraph, the second sentence was revised to report the correct dimensions of the column used for amino acid analysis, 150 × 4.6 mm (not 150 × 4 mm, as had been incorrectly reported).