

# Analysis of Isinglass Residues in Beer

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## ABSTRACT

*J. Inst. Brew.* 113(2), 130–134, 2007

A method has been developed for the detection of trace quantities of residual isinglass present in fined beers. Isinglass residues in beer are concentrated by means of an antibody raised using isinglass as an antigen. The separated isinglass is hydrolysed to its constituent acids and quantified by measuring the content of hydroxyproline. The limit of quantification of this method, that is the concentration of isinglass in beer which can be distinguished from background levels of hydroxyproline, is 0.17 mg of isinglass/litre of beer (on an isinglass dry weight basis). This method has been used to quantify the concentration of isinglass residues which could be present in brewery and cask conditioned beers, and also to determine whether there is likely to be stratification of isinglass residues in casks.

**Key words:** Analysis, beer, isinglass.

## INTRODUCTION

Allergic reactions to food components are becoming more common in developed societies. In order to protect consumers against unknowingly eating foods to which they are allergic, a number of countries across the world now require foods, including alcoholic beverages, to be labelled if they contain, or are made from or with, known allergens. The list of such allergens generally includes fish and fish products, thus beers which are clarified with isinglass (which is obtained from the swim bladders of certain tropical fish) would also need to be labelled. However, it is recognised that most of the isinglass protein reacts with beer components and is removed as a precipitate, leaving only trace amounts in the beer. In some countries, including the EU, it is possible for foods to be exempted from the labelling requirements if it can be proved that they do not pose a risk to susceptible individuals<sup>3</sup>. The risk of serious allergic reactions can be affected by the amount of residues of the allergen which remain in the food as consumed. It is therefore important to be able to measure accurately the amount of isinglass which might remain in beers.

Since the flocs formed by reaction of isinglass with yeast and beer proteins are removed from the beer by filtration, centrifugation or sedimentation, residues of isinglass in the beer as consumed by the customer are very low. Quantification is further complicated by the fact that, in addition to isinglass protein, beer also contains many

other proteins, thus detection of very small quantities of isinglass in beer is not straightforward. Since isinglass contains hydroxyproline, which is rare in other brewing materials such as cereals or hops, methods which rely on quantifying the amount of this imino acid in fined beer offer a potential mechanism for measuring isinglass residues in beer<sup>5</sup>. This paper describes a sensitive method which depends upon the use of antibodies specifically raised against isinglass. These antibodies are first added to the beer sample to extract and thus concentrate any isinglass residues. The actual amount of isinglass is then quantified by measuring the amount of hydroxyproline. A range of beers have been surveyed in order to determine typical and “worst case” concentrations of isinglass residues.

## MATERIALS AND METHODS

### Preparation and testing of the antibody

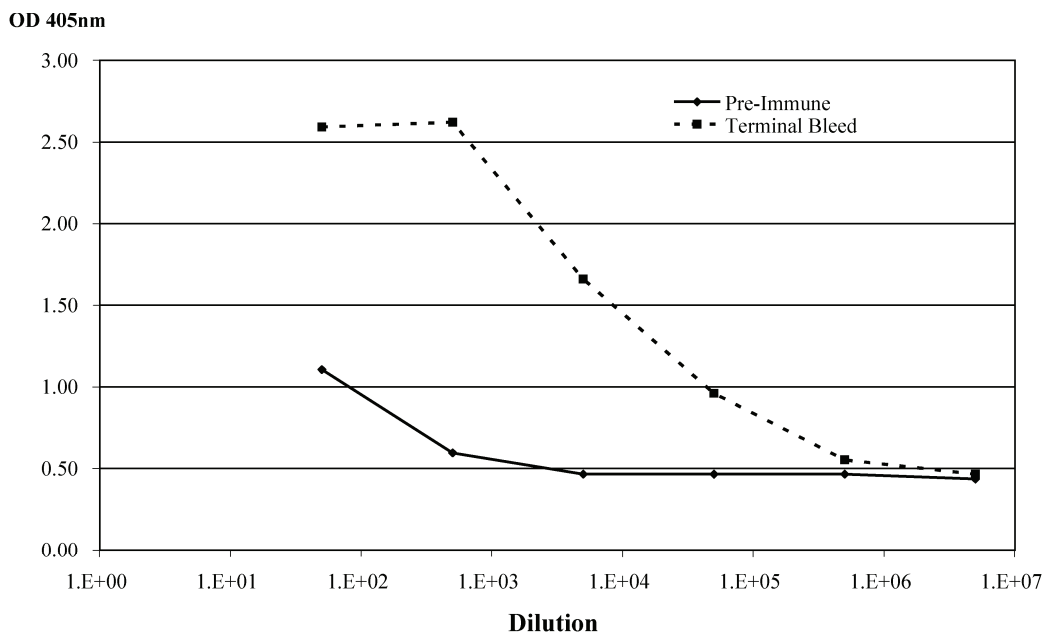
Polyclonal antibodies were raised in rabbits by the specialist company Biogenesis UK. The immunogens were commercial samples of pure isinglass (both powder and paste) provided by the major suppliers of commercial isinglass. Although processing details may vary between manufacturers, all commercial isinglass products are blends of material from the same limited range of tropical fish. Thus the isinglass samples used are expected to be representative of those marketed for fining beer. The antibodies provided were purified by ammonium sulphate precipitation prior to use. Serum was centrifuged at 12,000 rpm for 30 min at 4°C using a Beckman centrifuge. The supernatant was carefully poured off and the pellet discarded. Ammonium sulphate was slowly added to the supernatant to achieve a final concentration of 30% (saturation), and then stirred for 30 min at room temperature to encourage precipitation of IgG. The mixture was centrifuged at 10,000 rpm for 10 min at 4°C, then the supernatant was carefully removed and the pellet re-suspended in 10 mL of 0.9% saline (w/v). The ammonium sulphate precipitation procedure was repeated for a second time and the resulting pellet was re-suspended in 0.9% saline. The purified antibody solution was dialysed overnight in phosphate buffered saline, using dialysis tubing with a 3.5 kDa molecular weight cut-off. The antibody solution was stored at 4°C in the presence of 0.02% (w/v) sodium azide until required.

### Validation of antibodies for reactivity to isinglass

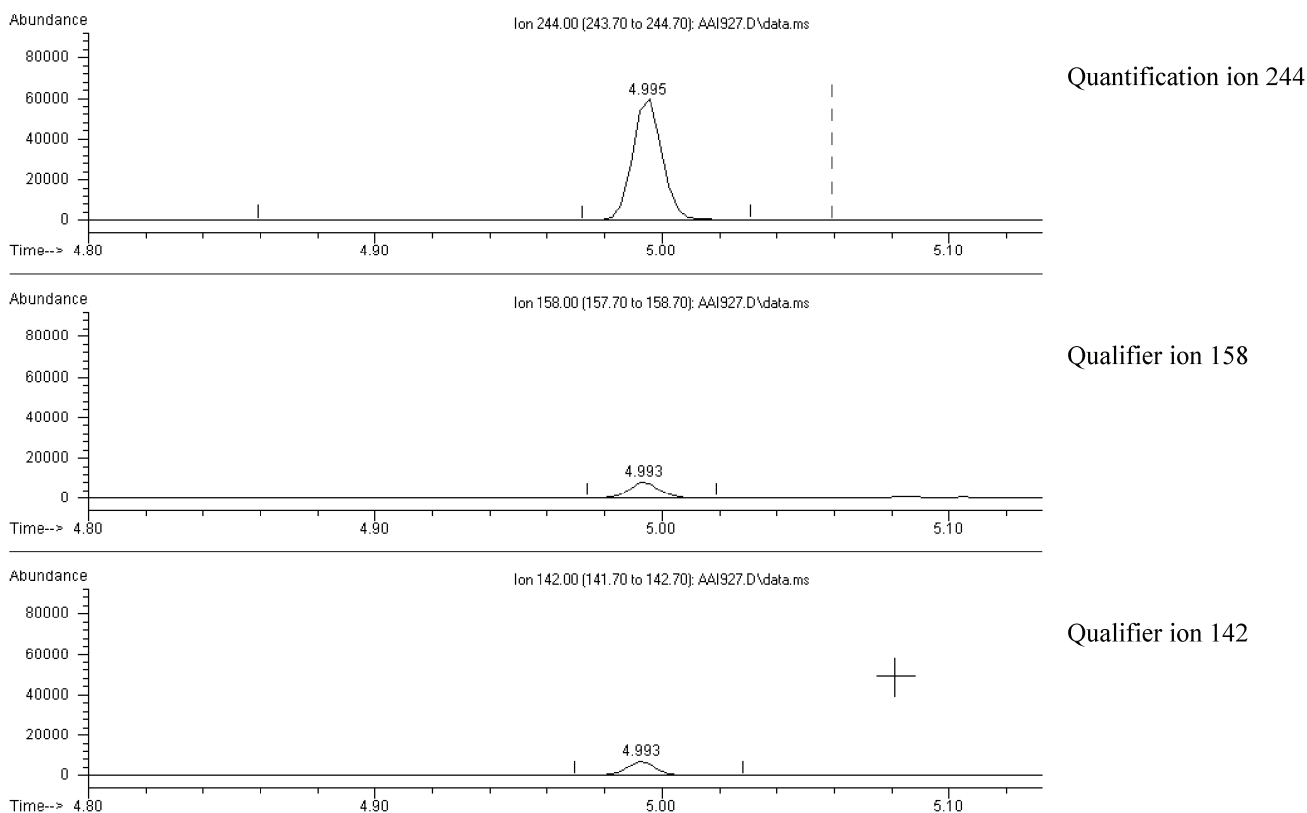
The antibodies were tested for their reactivity to isinglass using an ELISA protocol. ELISA plates were coated with 1 µg of isinglass, using 200 µL of a solution of isin-

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**Fig. 1.** Testing of purified antibody using ELISA.



**Fig. 2.** Selection ion chromatogram for 50 nmoles/mL hydroxyproline standard.

glass per well. Serial dilutions (10 fold) of the pre-immune and immune sera were prepared at the same time. The antisera were incubated in the treated wells at 37°C for 30 min. The reaction was visualised with an alkaline phosphatase-conjugated secondary antibody and para-nitrophenol phosphate at 405 nm. Fig. 1 shows the increase in absorbance associated with the immune serum when compared to the pre-immune serum. The greatest difference in reaction

was observed at the 1:500 dilution and therefore subsequent analyses was performed using this concentration of antibody.

### Separation of isinglass residues from beer

Degassed beer was adjusted to pH 7.0, using a magnetic stirrer with drop-wise addition of 3M NaOH, allowing any precipitated materials to re-dissolve. It was impor-

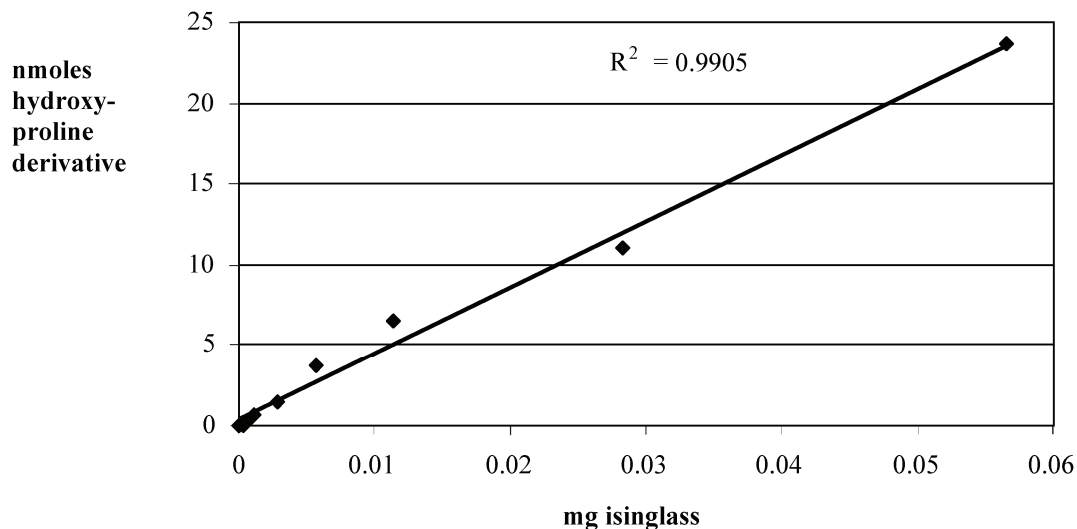


Fig. 3. Correlation of hydroxyproline concentration to isinglass content.

tant that this was done slowly and that the pH-adjusted beer should still be bright.

Antibody (200  $\mu$ L) was added to 100 mL of beer and the mixture was stirred gently at room temperature for 30 min. The antibody-antigen complex was then precipitated using ammonium sulphate added to 30% saturation. After stirring for a further 30 min, the precipitate containing the antibody-isinglass complex was collected by centrifugation (6500 rpm for 20 min). The supernatant was discarded and the pellet containing the antibody-isinglass complex was re-dissolved in 16 mL of 20 mM phosphate buffer pH 7. This was then dialysed for 20 h against deionised water (3 changes) using Spectropore CE dialysis tubing with a molecular weight cut off of 3.5 kDa (equivalent to approximately 25–30 amino acid residues) then lyophilised. Hydroxyproline content was then determined on these samples.

#### Hydrolysis of residues to constituent amino acids and derivatisation

Test samples were reconstituted and hydrolysed to their constituent amino acids by heating in a sealed vial with 50% HCl at 100°C for 4 h. Hydroxyproline and amino acids in the hydrolysate were derivatised and quantified using a Phenomenex EZ:faast™ kit, according to the manufacturer's instructions.

#### Quantification of hydroxyproline

Derivatised hydroxyproline was separated and quantified by gas chromatography with mass spectrophotometric detection. The GC-MS was fitted with a Phenomenex ZB-50 column and operated in selected ion monitoring mode. The quantification ion for hydroxyproline was  $m/z$  244, with 158 and 142 as qualifier ions (Fig. 2). Identification was confirmed from retention times and by matching the qualifier ion ratios with those of the authentic compound. Norvaline was used as the internal standard. Quantification was carried out using standard curves prepared by derivatisation of solutions of hydroxyproline over the range 0–200 nanomoles/mL. The limit of detection for hydroxyproline on the GC-MS was 40 pico-

moles/mL or 10 picograms injected. Allowing for the concentration of the beer during preparation of the samples, this corresponds to a limit of detection of approximately 1  $\mu$ g/litre of hydroxyproline in the beer, with a corresponding limit of quantification of 3  $\mu$ g/litre.

#### Correlation of hydroxyproline with isinglass

The values obtained from the GC-MS analysis were in terms of nanomoles of hydroxyproline. These had then to be converted to weights of isinglass. A calibration was developed by hydrolyzing known concentrations of isinglass and analyzing them for hydroxyproline as described above (Fig. 3).

#### Recovery

The recovery of isinglass from beer was estimated by adding a known weight of isinglass to beer produced in BRI's pilot brewery without the use of isinglass, then analyzing aliquots using the antibody procedure as previously described. At an addition rate of 2.26 mg isinglass (dry basis)/100 mL beer, the recovery ranged from 87–91.5%.

#### Survey of commercial small pack beers

Bottled and canned commercial beers were purchased in the UK in 2004/2005. Brands were chosen to include different beer styles (lagers, ales, stouts), pack types, and both large national/international and small regional producers. Beer known to be produced without isinglass (produced in BRI's pilot brewery) was used as a control beer.

#### Cask-conditioned beer

A commercial brand of ale known to use a high isinglass dose rate (72 mg/litre) was selected. A cask was set up in the BRI beer dispense cellar at 12°C, and allowed to settle for 3 days, as would be usual in commercial practice. The sampling protocol was designed to simulate a situation in a retail outlet in which beer is supplied to customers intermittently over several days. A one litre sample was taken via the tap and retained for analysis. The next day several litres were drawn off and a further sample taken for analysis. This was repeated over the next week

until the cask was almost empty and the beer sample was unacceptably cloudy and would not have been marketable. This sample represents the beginnings of the ullage. In addition, single samples were taken of a number of other brands of cask ale. In these cases the samples were taken when the casks were approximately half empty.

### Keg beer

A keg containing an international brand of brewery-conditioned beer was set up in the BRI cellar and sampled as described for the cask-conditioned ale, except that, since sedimented material had been removed by filtration in the brewery, the bottom samples remained clear and marketable.

## RESULTS

### Background levels of hydroxyproline in beer

In order to establish the background levels, if any, of hydroxyproline in beer, control beers were prepared in the BRI pilot brewery without using any isinglass. A total of eight samples, from 3 replicate all-malt brews, were taken and analysed for hydroxyproline using the antibody technique described. A mean concentration of 0.009 mg/litre of hydroxyproline was detected in the isinglass free control beer. This background level, which corresponds to 0.17 mg/litre of isinglass, was taken as the limit of quantification of isinglass in beer.

### Brewery-conditioned small pack beers

Table I lists the results for 13 commercial packaged beers, including 8 ales which were known to be clarified in the brewery using isinglass. Hydroxyproline could be detected in all of the samples except for three ales. When this was converted to isinglass equivalents, it can be seen that all the lagers and one of the stout samples were be-

**Table I.** Isinglass residues in commercial bottled and canned beers.

| Sample type  | mg hydroxyproline/<br>litre beer | mg isinglass/<br>litre beer<br>(dry weight basis) |
|--|----------------------------------|---|
| Isinglass-free control beer<br>(mean of 8 samples) | 0.009                            | 0   |
| Ale 1 (sample 1 – bottle)                          | 0.015                            | 0.3   |
| Ale 1 (sample 2 – can)                             | 0.014                            | 0.3   |
| Ale 2  | 0.021                            | 0.4   |
| Ale 3  | Not detected                     | Not detected                                      |
| Ale 4  | 0.011                            | 0.2   |
| Ale 5  | Not detected                     | Not detected                                      |
| Ale 6 (sample 1)                                   | 0.044                            | 0.8   |
| Ale 6 (sample 2)                                   | 0.029                            | 0.55  |
| Ale 6 (sample 2, replicate)                        | 0.015                            | 0.3   |
| Ale 7  | Not detected                     | Not detected                                      |
| Ale 8  | 0.020                            | 0.34  |
| Ale 8 (replicate)                                  | 0.007                            | < LOQ   |
| Lager 1  | 0.002                            | < LOQ   |
| Lager 1 (replicate)                                | 0.001                            | < LOQ   |
| Lager 2 (sample 1)                                 | 0.003                            | < LOQ   |
| Lager 2 (sample 2)                                 | 0.003                            | < LOQ   |
| Lager 2 (sample 3)                                 | 0.006                            | < LOQ   |
| Lager 3  | Not detected                     | Not detected                                      |
| Stout 1 (sample 1)                                 | 0.027                            | 0.5   |
| Stout 1 (sample 2)                                 | 0.005                            | < LOQ   |
| Stout 1 (sample 3)                                 | 0.034                            | 0.6   |
| Stout 2  | 0.039                            | 0.7   |

low the limit of quantification for isinglass. Although there was some variation between different samples, all contained less than 1 mg isinglass per litre of beer.

### Brewery-conditioned keg beer

Table II shows the concentration of isinglass residues found in a brewery-conditioned keg beer dispensed over a period of time. Concentrations were slightly higher than in small-pack beers, but the highest concentration found was below 3 mg/litre, with a mean of 1.0 mg/litre. Although there was some variation in isinglass concentrations, there was no evidence of any stratification of residues within the keg and there was no accumulation of isinglass residues at the bottom of the keg.

### Cask-conditioned beer

Table III shows the concentration of isinglass residues found in a cask-conditioned ale dispensed over a period of time. Within the bulk of the cask (that is, the beer which would be served to the customer) isinglass residues remained below 3 mg/litre, with a mean of 1.8 mg/litre and no evidence of stratification. The last sample which was drawn was noticeably hazy to the naked eye, and this contained a higher concentration of isinglass, 3.6 mg/litre. Samples drawn from below this point represented the ullage, and contained visible yeast and protein clumps. The

**Table II.** Isinglass residues in a brewery-conditioned keg beer.

| Sample      | mg hydroxyproline/<br>litre beer | mg isinglass/litre beer<br>(dry weight basis) |
|-------------|----------------------------------|---|
| Day 1       | 0.035                            | 0.6   |
| Day 2       | 0.155                            | 2.8   |
| Day 3       | 0.057                            | 1.0   |
| Day 4       | 0.112                            | 2.0   |
| Day 5       | 0.024                            | 0.4   |
| Day 8       | 0.035                            | 0.6   |
| Day 9       | 0.023                            | 0.4   |
| Day 10      | 0.064                            | 1.2   |
| Day 12      | 0.016                            | 0.3   |
| Day 17      | 0.036                            | 0.7   |
| Keg bottoms | 0.042                            | 0.8   |

**Table III.** Isinglass residues in a cask-conditioned ale.

| Sample       | mg hydroxyproline/<br>litre beer | mg isinglass/litre beer<br>(dry weight basis) |
|--------------|----------------------------------|---|
| Day 1        | 0.101                            | 1.9   |
| Day 2        | 0.140                            | 2.7   |
| Day 3        | 0.087                            | 1.7   |
| Day 4        | 0.104                            | 2.0   |
| Day 7        | 0.044                            | 0.9   |
| Day 7 – hazy | 0.187                            | 3.6   |

**Table IV.** Isinglass residues in cask ales.

| Sample  | mg hydroxyproline/<br>litre beer | mg isinglass/litre beer<br>(dry weight basis) |
|---------|----------------------------------|---|
| Brand 1 | 0.112                            | 2.1   |
| Brand 2 | 0.029                            | 0.6   |
| Brand 3 | 0.014                            | 0.3   |
| Brand 4 | 0.117                            | 2.2   |
| Brand 5 | 0.084                            | 1.6   |
| Brand 6 | 0.219                            | 4.2   |
| Brand 7 | 0.014                            | 0.3   |

concentration of isinglass at this level was very high (>100 mg/litre) but since it was associated with the solid material, the distribution was non-homogeneous and difficult to determine with any accuracy.

Samples were also taken from a number of other brands of cask ale, including one micro-brewery brand, and tested for isinglass residues. These were taken from casks approximately half empty. Results are shown in Table IV. Concentrations of isinglass in these 7 brands were similar to those found in the stratification study, with the highest concentration found being 4.2 mg/litre. This suggests that the cask chosen for the stratification study was representative of other brands of cask ale.

## DISCUSSION

The studies reported here indicate that the overwhelming majority of the isinglass added to beer as a fining agent is either removed in the brewery (for brewery-conditioned beers) or is contained in the sediments at the bottom of the cask (for cask-conditioned beers). In each case, the concentrations of isinglass contained in the beer marketed and consumed by the drinker are extremely low. Precise quantification of the residues which could be consumed is complicated by the presence of a wide range of other proteins, by the likely heterogeneous nature of the isinglass residues themselves and by the fact that the levels are close to the limits of quantification. These complications undoubtedly reduce the analytical precision.

Only a few other attempts to quantify isinglass residues in beer have been reported. A group at Heriot-Watt University have used determination of hydroxyproline as a method of quantifying isinglass residues in beer<sup>1</sup>. This group, who were not using any concentration techniques, found that although hydroxyproline could be detected in cask beers immediately after the finings were added, the levels fell to below the limit of detection once the beer had settled (typically 2–3 days).

A method, understood to be based on the classic biochemical principles of precipitation of the protein by ammonium sulphate, followed by separation of the protein molecules on the basis of size and electrical charge, has been developed in New Zealand<sup>4</sup>. This is based on the classic biochemical principles of precipitation of the protein by ammonium sulphate, followed by separation of the protein molecules on the basis of size and electrical charge. In order to achieve the desired sensitivity, large quantities (1–2 litres) of beer must be used for the assay. No results have been published using this method, but they are reported to be similar or lower than those found in the current study for small-pack brewery conditioned beers (*personal communication*). One concern with any method based on molecular size is that it runs the risk of missing collagen fragments which may (but also may not) have the same or similar allergenic potential to the parent molecule. One advantage of the antibody method used in the present study is that both parent molecule and larger fragments should be detected.

As previously noted, hydroxyproline is generally considered to occur at significant levels only in animal collagen. However, there are published reports in the scientific

literature of glycoproteins containing hydroxyproline in higher plants<sup>2,6</sup>. The current study did detect low levels of hydroxyproline in beer which had not been treated with finings. This has not been previously reported, probably because methods have not been sufficiently sensitive. If barley contained such glycoproteins, any fragments remaining in the beer would have been concentrated by the extraction procedure used in the study reported here. Thus the background levels of hydroxyproline detected in unfinned beer are a likely consequence of the low limit of detection of the method used.

In the current study, cask beer was sampled on a daily basis, over a total time period of one week, with the final samples being drawn from close to the sedimented material, where they appeared visibly cloudy. In fact, the flavour of a cask beer will begin to deteriorate if it is not consumed within 3–4 days after tapping, and in commercial practice casks may be replaced before all the marketable beer has been consumed. Thus the maximum concentrations detected in this study are likely to represent a “worst case” as far as dietary exposure for the consumer is concerned.

## CONCLUSIONS

The results of the study reported here confirm that the concentration of residues remaining in beers fined with isinglass is indeed very low. For many bottled and canned beers, levels of isinglass are below the limit of quantification. Slightly higher concentrations may be found in beers in large containers such as kegs and casks, but even here the maximum concentrations would be likely to remain below 5 mg/litre. There is no evidence of significant stratification of isinglass residues in the marketable beer in such bulk containers.

## ACKNOWLEDGEMENTS

The authors are grateful to the IBD Charitable Trust, who provided part of the funding for this work.

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(Manuscript accepted for publication June 2007)