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Role of Riboflavin in Beer Flavor Instability: Determination of Levels of Riboflavin and Its Origin in Beer by Fluorometric Apoprotein Titration

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A method for the quantitative determination of riboflavin levels in beer was developed. The method is based on the quenching of riboflavin fluorescence, which occurs when riboflavin binds to the aporiboflavin-binding protein from egg white. The method does not require any pretreatment of the beer before analysis, other than dilution, and proved to be simple, reliable, and sensitive. The lowest concentration that could be detected was ∼10 nM riboflavin. The possible interference of flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD) with the determination of the riboflavin content of beer was excluded, because beer contains only a very small amount of FAD (0.03 μM) and no FMN. The riboflavin levels of the types and brands of beer investigated were in the range of 0.5−1.0 μM. The origin of the riboflavin in beer proved to be the malt. Hop and yeast hardly contributed to the riboflavin content of beer. Besides its use in the determination of riboflavin levels, the aporiboflavin-binding protein also provides a way to remove riboflavin from beer, which reduces the light sensitivity and the related lightstruck off-flavor formation in beer.

KEYWORDS: Beer; riboflavin levels; fluorescence; egg white riboflavin-binding protein

INTRODUCTION

Riboflavin has for a long time been implicated as a photosensitizer in the formation of sunstruck flavor in beer (*1*−*4*). In addition, it has been postulated that apart from the role in sunstruck flavor formation, riboflavin and other flavins (*5*−*7*) are also involved in the formation of reactive oxygen species in beer and thus contribute to the formation of stale flavor in general. Hence, the photosensitizing properties of flavin entities have a negative impact on the stability of beer flavor and, consequently, the selective removal of these flavins from beer could be a strategy toward improving the robustness and quality of beer flavor (*8*). In this respect it is of interest to know the concentration of riboflavin in beer. The classical procedures for measurement of the riboflavin concentration in biological samples include fluorometric, enzymatic, microbiological, chromatographic, and radioisotopic methods. These techniques are time-consuming and/or subject to interference by unknown factors (*9*−*11*). A simple method, successfully applied in the determination of the riboflavin concentration of urine (*12*, *13*), is based on the binding of riboflavin to the riboflavin-binding protein (RfBP) and the concomitant decrease of the riboflavin fluorescence.

RfBP from egg white is a globular monomer of ∼30 kDa (*14*). Apo-RfBP binds riboflavin in a 1:1 ratio, with high affinity: K_d = 1.3 nM (at pH 7.0, 25 °C). The binding of riboflavin to apo-RfBP is nearly independent of pH between pH 6 and 9 but rapidly declines with decreasing pH below pH 6 (*15*, *16*). At the pH of beer, which is ∼4.2, the K_d amounts to ∼1.6 μM (*8*). The binding of riboflavin to apo-RfBP almost completely quenches riboflavin fluorescence (*13*, *17*), due to stacking of the riboflavin isoalloxazine ring with aromatic side chains in the riboflavin binding site of RfBP (*14*, *18*).

Although the role of riboflavin in beer flavor change, particularly in the case of sunstruck, is well documented, it is not clear how flavin levels vary in beer or whether the flavin-mediated flavor change is correlated with the flavin level. The purpose of this paper is to investigate what levels of riboflavin are found in a range of typical production beers and to determine the source of riboflavin in beer.

MATERIALS AND METHODS

Reagents, Proteins, and Beer Samples. Riboflavin, flavin mononucleotide (FMN), and flavin adenine dinucleotide (FAD) were obtained from Sigma. A stock solution of 100 μM riboflavin in water was stored in the dark at room temperature. Every 2 weeks a fresh riboflavin stock solution was prepared, and the riboflavin concentration (and thus the stability) of the solution was checked every day by measurement of the absorbance: ϵ_{450} = 12200 $M^{-1}\cdot cm^{-1}$. The concentrations of FMN

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Table 1. Riboflavin Content of Different Brands of Beer

brand	[riboflavin][a] (μM)	*n*
A	0.81 ± 0.05	4
B	0.77 ± 0.01	7
C	0.83 ± 0.05	7
D	0.81 ± 0.02	7
E	0.56 ± 0.01	5
F	0.71 ± 0.03	7
G	1.00 ± 0.10	7
H	0.92 ± 0.17	12

[a] The riboflavin concentration is the average of *n* determinations ± the standard deviation, performed as described under Materials and Methods. Dilution of the beers: A–F, 300 μL; G, 100 μL; H, 15 μL; was added to 50 mM NaP_i, pH 7.0, to a final volume of 1 mL. A–F were lager beers, containing 5% alcohol; G and H were high-gravity dark beers; G contained 5% and H 8% alcohol.

Table 2. Riboflavin in Sweet Wort and Beer

sample	[riboflavin][a] (μM)	*n*
sweet wort	1.41 ± 0.13	4
unhopped beer	0.81 ± 0.02	5
hopped beer	0.80 ± 0.02	5

[a] The riboflavin concentration is the average of *n* determinations ± the standard deviation, performed as described under Materials and Methods. Dilution of the samples: sweet wort, 100 μL; hopped and unhopped beer, 300 μL; was added to 50 mM NaP_i, pH 7.0, to a final volume of 1 mL. The sweet wort did not contain hop; the hopped and unhopped beers were identical except for the presence or absence, respectively, of hop.

and FAD stock solutions were calculated from their absorbance at 450 nm: $\epsilon_{450} = 12200\ M^{-1}\cdot cm^{-1}$ and $\epsilon_{450} = 11300\ M^{-1}\cdot cm^{-1}$, respectively (*19*). The solutions of FMN and FAD were also kept in the dark at room temperature. NADPH was obtained from Boehringer Mannheim.

RfBP was purified from egg white, and apo-RfBP was prepared as described by Miller and White (*17*). The apo-RfBP concentration was calculated from the absorbance at 280 nm: $\epsilon_{280} = 49000\ M^{-1}\cdot cm^{-1}$ (*17*). Apo-RfBP stock solutions (5 mg/mL, in water) were stored at −20 °C. Bovine serum albumin (BSA) was obtained from Sigma. BSA stock solutions (10 mg/mL in water) were stored at −20 °C. *p*-Hydroxybenzoate hydroxylase (PHBH) from *Pseudomonas fluorescens* was a gift from Dr. W. van Berkel (Laboratory of Biochemistry, Wageningen University). *p*-Hydroxybenzoate (PHB) was obtained from Aldrich.

Beers A−G (**Table 1**) were bought in the supermarket; the sweet wort and the hopped and unhopped beers used for **Table 2**, as well as beer H (**Table 1**), were obtained from Quest International.

Fluorescence Measurements. Fluorescence was measured with a Fluorolog-3 spectrofluorometer (FL3-22) from Instruments S.A., Inc., Jobin−Yvon Spex, France/USA: excitation wavelength, 450 nm (band-pass 0.5 nm); emission wavelength, 525 nm (band-pass 5.0 nm). All measurements were performed at room temperature.

Illumination of Beer. Lager beer, kept in a green glass bottle, was illuminated in a self-made wooden box (size 30 × 30 × 30 cm) equipped with an 8 W TL tube (DAKS), type cool white. The intensity of the light, measured inside the empty bottle (with a MX4 lux meter from BBC Goerz Metrawatt), was 4500 lx. During illumination the temperature was kept at 30 °C. Samples of the beer could be taken aerobically, because oxygen does not degrade riboflavin. After a sample was taken, the bottle was closed again (with Parafilm) and put back into the light box for further illumination.

Determination of FAD Concentration. The concentration of FAD in beer was determined from the rate of oxidation of NADPH by PHBH, during catalysis of the conversion of POHB, as described by Müller and van Berkel (*20*). After 2.5 min of incubation of apo-PHBH with 0.2 mM POHB + 0.2 mM NADPH in 0.1 mM Tris-HCl, pH 8.0, at 25 °C, the reaction was started by addition of FAD or beer. The oxidation of NADPH was monitored at 340 nm. The FAD concentration of beer was determined from a calibration curve (rate of NADPH oxidation vs FAD concentration), obtained by addition of aliquots of a standard FAD solution to the reaction mixture.

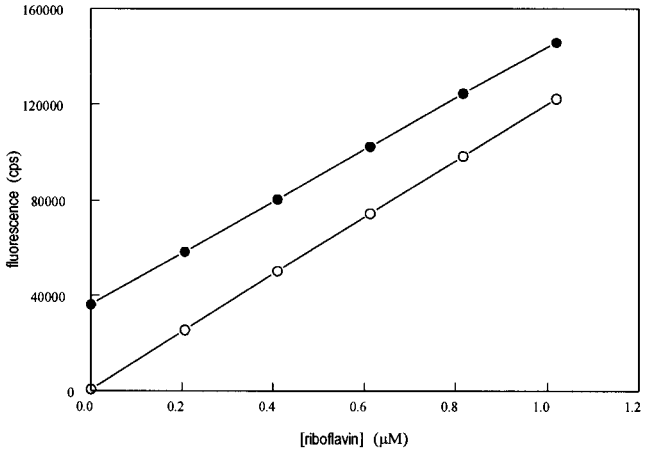

Figure 1. Titration of riboflavin in 50 mM NaP_i, pH 7.0: (○) without beer; (●) with beer [0.3 mL of beer (brand E, **Table 1**) in 1 mL total volume]. Riboflavin was added from a 100 μM stock solution. The riboflavin fluorescence was measured as described under Materials and Methods; fluorescence units are counts per second (cps).

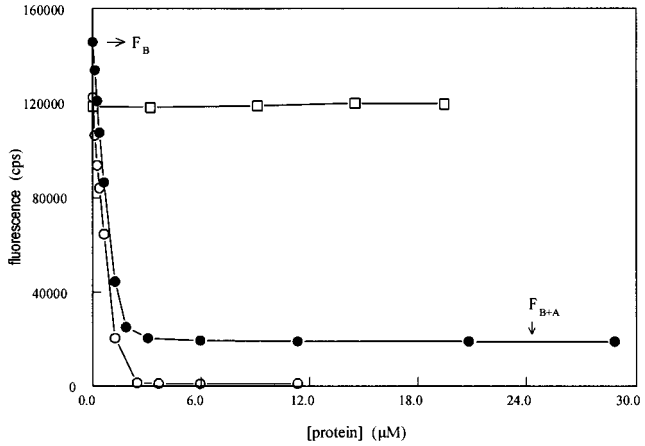

Figure 2. Titration of apo-RfBP (○ and ●) and BSA (■) in solutions of riboflavin. Solutions ○ and □ contained 1.0 μM riboflavin + 50 mM NaP_i, pH 7.0; solution ● contained 0.3 mL of beer (brand E, **Table 1**) + 1.0 μM riboflavin + 50 mM NaP^i, pH 7.0, in 1 mL total volume. Apo-RfBP and BSA were added from 5 and 10 mg/mL stock solutions, respectively. The riboflavin fluorescence was measured as described under Materials and Methods. F_B is the fluorescence of the beer sample + the riboflavin added to the sample in order to obtain the calibration curve (**Figure 1**); F_{B+A} is the fluorescence of the beer/riboflavin mixture after full titration with apo-RfBP.

Riboflavin Assay. Samples of beer were diluted in 1 mL of 50 mM NaP_i, pH 7.0 (final concentration), to such extent that the absorbance values of the beer samples at the excitation wavelength, 450 nm, were in the range of 0.05−0.07. The samples were kept in the dark until analysis. For each beer sample a riboflavin calibration curve (fluorescence intensity vs riboflavin concentration, like **Figure 1**) was prepared by adding aliquots of the 100 μM riboflavin stock solution to the beer, followed by measurement of the fluorescence. Subsequently, aliquots of apo-RfBP were added to this beer/riboflavin mixture, and the fluorescence was measured again, after each addition (like **Figure 2**). This was done until no further decrease in the fluorescence of the beer sample was observed.

The riboflavin concentration of the beer (R_B) was calculated with the equation

$$R_B = \{[(F_B - F_{B+A})/S] - R_C\} \times D \quad (1)$$

where R (μM) is the riboflavin concentration, R_B (μM) is the riboflavin concentration of the beer, R_C (μM) is the amount of riboflavin added to the sample to obtain the calibration curve, F (cps) is fluorescence, F_B is the fluorescence of the beer sample plus the riboflavin added to the sample to obtain the calibration curve, F_{B+A} is the fluorescence of the beer/riboflavin mixture after full titration with apo-RfBP, S (cps/μM) is the slope of the calibration curve, and D is the factor by which the beer was diluted. The difference in the fluorescence of the beer sample plus added riboflavin, before addition of apo-RfBP (F_B) and after the final addition of apo-RfBP (F_{B+A}), obtained from the apo-RfBP titration curve (**Figure 2**), divided by the slope, S, of the riboflavin calibration curve (**Figure 1**) yields the total riboflavin concentration of the diluted beer sample plus added riboflavin ($R_B + R_C$). Subtraction of the concentration of the riboflavin added to the beer sample to obtain the calibration curve (R_C) yields the riboflavin concentration of the diluted beer sample. Finally, multiplication with the factor by which the beer was diluted, D, yields the riboflavin concentration of the original beer, R_B.

RESULTS AND DISCUSSION

Figure 1 shows that the relationship between the fluorescence intensity and the riboflavin concentration of a (pH 7.0) solution is linear, both in the absence and in the presence of beer, irrespective of the brand. The slope (S, eq 1) of the curve is independent of the pH of the riboflavin solution (pH 4−7, data not shown). In the beer sample the slope is a little less steep, which indicates that (unidentified) compounds in beer quench the riboflavin fluorescence to some extent. However, because the curve is linear, a fluorescence change can be correlated to a corresponding change in riboflavin concentration.

Addition of apo-RfBP to a riboflavin solution causes quenching of the riboflavin fluorescence. This is illustrated in **Figure 2** for a standard riboflavin solution and for a beer sample (both of pH 7.0), to which equal amounts of riboflavin were added. Addition of BSA instead of apo-RfBP to the riboflavin standard or the beer sample did not decrease the fluorescence (**Figure 2**), which proves that the observed fluorescence decrease upon addition of apo-RfBP is caused by specific binding of riboflavin to apo-RfBP and not merely by the presence of protein. Each solution was titrated with apo-RfBP until the fluorescence did not further decrease. Unlike the situation in buffer, where the fluorescence was quenched almost entirely, a certain level of fluorescence remained in the beer sample, even when an excess of apo-RfBP was added (**Figure 2**). This background fluorescence level (F_{B+A}, eq 1) did not change in time (while the sample was kept in the dark for 3 h, data not shown). The background fluorescence level indicates the presence of other compounds in beer that have fluorescence characteristics similar to those of riboflavin but which do not bind to apo-RfBP. Because these compounds do not bind the apoprotein, their presence does not hamper the determination of the riboflavin concentration in beer by the above-described method.

The linear relationship between fluorescence intensity and the amount of riboflavin added (S, eq 1, from **Figure 1**) allows calculation of the riboflavin concentration of the beer (R_B, eq 1) from the difference in the fluorescence intensity before addition of apo-RfBP (F_B, eq 1, from **Figure 2**) and after full titration with apo-RfBP (F_{B+A}, eq 1, from **Figure 2**).

At pH 7.0, apo-RfBP binds riboflavin instantaneously and with high affinity. At the pH of beer, which is ~4.2, the dissociation constant for the binding of riboflavin to apo-RfBP is about a factor of 1000 higher than at pH 7.0, according to Becvar and Palmer (*16*). **Figure 3** shows the quenching of riboflavin fluorescence due to the binding of riboflavin to apo-RfBP at pH 7.0 and at pH 4.2, both in the presence of beer. The height of the background fluorescence level, which remains after full titration with apo-RfBP, is independent of the pH of the solution. More apo-RfBP must be added at acidic pH than at neutral pH in order to reach the background fluorescence level, but, clearly, the affinities of apo-RfBP for riboflavin do not differ as much as expected from the literature data. From **Figure 3** a $K_d = 0.29$ μM could be calculated for the binding of riboflavin to apo-RfBP at pH 7.0, and at pH 4.2 a value of 0.62 μM was calculated for the dissociation constant. Especially at pH 7.0 the value obtained for the dissociation constant is much larger than the value given by Becvar and Palmer (*16*), which was 1.3 nM. A difference in ionic strength might be the cause of the larger dissociation constant: Becvar and Palmer (*16*) acquired their data in 10 mM KP_i, whereas the data in **Figure 3** were obtained in 50 mM NaP_i and in the presence of beer. Ethanol, even at 1 M concentration (as in lager beer), does not influence the apo-RfBP−riboflavin binding (data not shown).

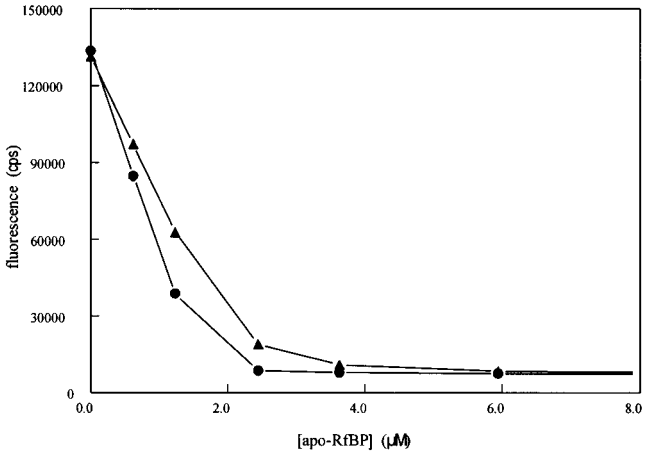

Figure 3. Titration of apo-RfBP in solutions containing beer (brand E, **Table 1**), at pH 7.0 (●) and pH 4.2 (▲). Both solutions contained 1.0 μM riboflavin + 0.1 mL of beer in 1 mL (total volume); in addition solution ● contained 50 mM NaP_i, pH 7.0, and solution ▲ contained 50 mM NaAc/HAc, pH 4.2. The riboflavin fluorescence was measured as described under Materials and Methods.

As remarked above, the background fluorescence level observed in beer after full titration with apo-RfBP (**Figures 2** and **3**) indicates that there are compounds in beer which have the same fluorescence characteristics as riboflavin but which do not, or hardly, bind to apo-RfBP at pH 4.2 or 7.0. Other flavins, such as the riboflavin derivatives FMN and FAD, have fluorescence properties similar to those of riboflavin. At pH 7.0 these flavins are bound by apo-RfBP, although with a much lower affinity than riboflavin: at 25 °C, $K_d = 1.4 \times 10^{-6}$ M for the binding of FMN and $K_d > 1.4 \times 10^{-5}$ M for the binding of FAD (*16*). The higher affinity of apo-RfBP for riboflavin compared to any other flavin compound does not exclude the possibility that in the determination of the riboflavin concentrations in beer samples (**Tables 1** and **2**), besides riboflavin, also other flavins were bound to apo-RfBP. Andrés-Lacueva et al. (*21*) determined riboflavin, FMN, and FAD concentrations in beer by HPLC measurements. According to their data, a typical lager beer (such as brand A, **Table 1**) contained 0.77 μM riboflavin, 0.06 μM FAD, and no FMN. The concentration of FAD in a solution can be determined not only by HPLC but also from the rate of the oxidation of NADPH during the conversion of *p*-hydroxybenzoate by the apo-form of the enzyme *p*-hydroxybenzoate hydroxylase, which requires FAD as a prosthetic group. Using this method (*20*), we found that lager beer of brand A (**Table 1**) contained 0.025 ± 0.004 μM FAD

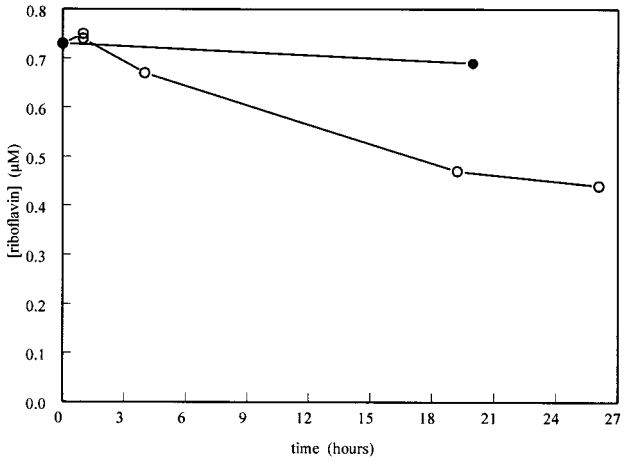

Figure 4. Decrease of the riboflavin concentration of a lager beer (brand A, **Table 1**), packed in a green glass bottle, during illumination (○) and in the dark (●). During the experiment beers ○ and ● were kept at 30 °C. The illumination and the determination of the riboflavin concentration of the beer were performed as described under Materials and Methods.

(average of four determinations). In neutral aqueous solution the fluorescence quantum yield of both riboflavin and FMN is ~9 times higher than that of FAD (*22*), but with decreasing pH the FAD fluorescence becomes more intense, due to dissociation of the intramolecular complex between the isoalloxazine ring and adenine moiety. At pH 4.0 the FAD fluorescence intensity is ~3.5 times lower than the intensity of the riboflavin fluorescence, and the binding of FAD to apo-RfBP is stronger (data not shown). Because the apo-RfBP titration's were performed at pH 7.0, in the case of beer A (**Table 1**) ~0.4% of the fluorescence attributed to riboflavin actually could have been a contribution of the FAD present in the beer. This is well within the error margin of the data given in **Table 1**. From these considerations we conclude that FMN and FAD do not interfere with the fluorometric riboflavin determination and that this method gives reliable data for the riboflavin concentration in beer. Furthermore, it is concluded that the background fluorescence level which remains after full titration of a beer sample with apo-RfBP (**Figures 2** and **3**) does not originate from FMN, because this is not present in beer, nor from FAD, because, especially at pH 7.0, the fluorescence intensity of FAD at such a low concentration as found in beer is too low to account for the height of the background level.

Riboflavin acts as a photosensitizer in beer, thereby initiating degradation of beer components. At the same time, riboflavin is itself decomposed by the influence of light (*23*). **Figure 4** shows the decrease of the riboflavin content of a lager beer, packaged in a green glass bottle, during illumination, determined by the fluorometric riboflavin assay. After ~25 h of illumination, the riboflavin concentration was about two-thirds of the concentration before illumination. Hardly any degradation of the riboflavin concentration was observed when the beer was kept in the dark (**Figure 4**). At the pH of beer the main light-induced degradation product of riboflavin is lumichrome. This alloxazine compound is fluorescent and also acts as a photosensitizer (*24*). Lumichrome is bound by apo-RfBP, with $K_d = 9.3 \times 10^{-8}$ M at pH 7.0 and 25 °C (*16*), and might thus interfere with the fluorometric riboflavin determination if photodecomposition of riboflavin takes place (as in **Figure 4**). However, lumichrome has different absorbance and fluorescence characteristics than riboflavin and does not absorb at 450 nm (*23*). This also implies that the fluorescence background level in a beer sample (**Figures 2** and **3**) is not caused by lumichrome, which might be present in the beer.

As stated earlier, the presence of unidentified compounds in beer that cause a background level of fluorescence to remain, even in the presence of an excess of apo-RfBP (**Figures 2** and **3**), does not obstruct the determination of the riboflavin content of beer by the fluorometric titration with apo-RfBP. Therefore, the nature of these compounds was not further investigated.

The riboflavin content of several brands of beer was determined by the fluorometric titration with apo-RfBP (**Table 1**). Beers A−F were lager beers of different brands, and beers G and H were high-gravity dark beers. Beers A−G contained 5% alcohol; beer H contained 8% alcohol. Except for beer E, the lager beers contained similar levels of riboflavin, which are in agreement with the riboflavin content of a typical lager beer, determined (with HPLC) by Andrés-Lacueva et al. (*21*). The riboflavin content of beer E was significantly lower than that of the other lagers, probably because this was a lager brewed with substantial amounts of adjunct (alternative sources of starch, added to the malt). The dark beers G and H contained a significantly higher amount of riboflavin than the lager beers. Beer H required a much greater dilution, 67 times, than the lager beers, because of the extremely dark color of the beer. This illustrates the sensitivity of the method: the riboflavin concentration actually determined (in the diluted beer) was as low as 14 ± 4 nM, but the standard deviation from the average was high, 18%, probably because of small errors in the dilution of the beer.

Possible sources of riboflavin in beer are the malt, hop, and yeast. The sweet wort (**Table 2**) contained a relatively high amount of riboflavin, as compared to the riboflavin content of beer (**Table 1**). This can be explained by dilution effects during sparging (the washing out of sugars from the filter bed) and, to a lesser extent, by dilution while bringing the final product within specifications. Special pilot brews of unhopped and hopped lager beer were used for the determination of the contribution of hop to the riboflavin content of beer. **Table 2** shows that unhopped beer had the same riboflavin content as hopped beer. During fermentation of the wort, yeast (*Saccharomyces cerevisiae*) produces 50−100 μg of riboflavin/L (dry weight), which is retained within the cells (*25*). The maximum biomass production is 4−6 g of yeast cells/L, of which ~1 g/L is inactive cells; 10−20% of the inactive cells leak (*26*), which may cause release of riboflavin into the beer. From these data follows it that the maximum contribution of yeast to the riboflavin content of beer is ~50 nM. The high riboflavin content of the sweet wort sample, the absence of an effect of hop, and the minor contribution of yeast to the riboflavin concentration show that the riboflavin in beer originates from the malt.

Apo-RfBP might be applicable in the removal of riboflavin from beer at some stage in the brewing process. Addition of this protein to beer is likely to block the detrimental activity of riboflavin (as exemplified by the fluorescence data in this study), which would minimize radical-related off-flavor formation in beer.

ABBREVIATIONS USED

(apo-)RfBP, (apo)riboflavin binding protein; cps, counts per second; eq, equation; FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide; HPLC, high-performance liquid chromatography; K_d, dissociation constant; *n*, number of experi-

ments; NADPH, nicotinamide adenine dinucleotide phosphate (reduced); PHBH, *p*-hydroxybenzoate hydroxylase; SD, standard deviation.

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