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Spectrophotometric Method for Exploring 3-Methyl-2-butene-1-thiol (MBT) Formation in Lager

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The disappearance of riboflavin absorbance at 445 nm from beers or model beers on light exposure is directly linked to light-struck character formation. The addition of (+)-catechin, (−)-epicatechin, tryptophol, or ascorbic acid was able to reduce, but not stop, absorbance loss or light-struck character formation in either model beer or mainstream lager that was exposed to light. When isohumulone was present in model beer, the inhibitory effect of (+)-catechin, (−)-epicatechin, or tryptophol decreased with increasing isohumulone. The spectrophotometric method used in this study is a simple and effective method for determining light-struck susceptibility.

KEYWORDS: Light-struck character; 3-methyl-2-butene-1-thiol; MBT formation; riboflavin; spectrophotometric method

INTRODUCTION

Light-struck (sun-struck or "skunky") flavor is one of the flavors most disliked by beer drinkers (*1*). When beers are exposed to visible light, an objectionable skunky aroma known as light-struck character is formed in most. This aroma is attributed principally to the formation of 3-methyl-2-butene-1-thiol (MBT) (*2*). MBT is produced when isohumulones react with sulfur-containing compounds in the presence of light. The isopentyl side chain of isohumulones is a unique requirement for MBT formation (*3*). However, as isohumulones do not absorb light in the region known to trigger MBT formation (350−500 nm), MBT production must depend on a photosensitizer. Riboflavin is present in beer at a concentration of 0.2−1.3 mg/L (*4*, *5*). Flavins absorb visible light and may transfer energy to isohumulone and sulfur compounds. MBT is not formed in the absence of riboflavin. In model systems containing isohumulones and cysteine, Sakuma et al. showed that the concentration of MBT increased linearly with an increase in riboflavin concentration (*6*). MBT inhibitors such as 1,8-cineole compounds (*7*), gallotannins (*8*), polyphenols (*9*), and thioredoxin (*10*) have been mentioned in the literature. However, the effects of these inhibitors on light-struck character formation in light-exposed beers were not elucidated.

The low flavor threshold of MBT, coupled with its low concentration in beer, presents a serious challenge to its detection and quantification by modern instrumentation. This challenge is exacerbated by its high boiling point and its chemical reactivity (*2*). There are reports of the analysis of MBT using headspace gas chromatography (*11*, *12*), gas chromatography and mass spectrometric detection (*13*), solid-phase microextraction and gas chromatography with pulsed flame photometric detection (SPME-PFPD) (*14*), and gas chromatography−mass spectrometry (GC-MS) (*15*). However, these are time-consuming and expensive techniques.

The objective of this study was to evaluate the use of a simple spectrophotometric method to test for light-struck character formation on the basis of color loss of riboflavin in light-exposed beer/model beer. The effect of (+)-catechin, (−)-epicatechin, isohumulone, riboflavin, tryptophol, and ascorbic acid on the disappearance of riboflavin absorbance from lager and model beer was also investigated using this simple method. We conclude that the spectrophotometric measurement of riboflavin in beer is a useful and practical means of determining the susceptibility of beer to light-struck character development and for determining the inhibitory effects of different compounds on the stability of beer.

MATERIALS AND METHODS

Materials. (+)-Catechin, (−)-epicatechin, and tryptophol were supplied by Sigma-Aldrich, and ascorbic acid was supplied by BDH. All reagents were of AR grade. Lager and aqueous isohumulone solutions (30% w/v of their potassium salts) were supplied by Carlton & United Breweries (CUB, now Foster's Australia). The HP6 solution was manufactured by CUB in a process involving liquid carbon dioxide extraction of humulones followed by isomerization to the desired isohumulones.

Preparation of Model Beer. Model beer consisting of a 0.01 M citrate/phosphate buffer (pH 4.2), with various amounts of riboflavin, 25 mg/L isohumulones, 5% ethanol, and 20 mg/L cysteine (*16*) was prepared as follows immediately before each experiment.

Tripotassium citrate (0.3 g) was dissolved in 900 mL of deionized water and the pH adjusted to 4.2 with orthophosphoric acid. The

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solution was sparged with nitrogen for 30 min, followed by the addition of 20 mg of L-cysteine (HCl salt). Riboflavin working solution (20 mL of 25 mg of riboflavin in 100 mL of nitrogen-flushed deionized water) was added to the buffer solution along with 50 mL of 96% ethanol. Finally, 5 mL of isohumulone working solution (1/60 dilution of 30% HP6 solution) was added. The volume of the buffer solution was adjusted to 1 L with deionized water, and a further 30 min of purging with nitrogen was performed before this "model beer" was used for light-struck experiments. In some experiments the riboflavin concentration was varied from 1 to 10 mg/L. Additives were injected (100 μL) as solutions in ethanol or water with a microsyringe through the silicone septum into the beer.

Light Exposure Conditions of Real and Model Beers. All light exposure experiments were performed with illumination by an OSRAM L 58W/11-860 Lumixplus Tageslicht fluorescent tube. Samples were exposed to light of intensity 526, 3580, and 4580 lx (Hagner Digital Luxmeter 100). The fluorescent tube emission covered the visible spectrum from 350 to ≈700 nm, with maxima at 406, 436, 546, and 578 nm. Real or model beer samples were prepared by filling 20 mL clear glass gas chromatography vials and sealing the vials with crimp caps and silicone septa under a nitrogen atmosphere in an anaerobic chamber (Coy Laboratories model AC00 1901).

Spectrophotometric Determination of Riboflavin Absorbance. Riboflavin concentration was determined spectrophotometrically with a Beckman DU640 UV−vis spectrophotometer at 445 nm.

Sensory Analysis of Light-Struck Beer. Sensory analysis of samples for light-struck character was carried out by several experienced testers who had been trained in sensory evaluation at Carlton & United Breweries. The panelists used triangular testing, smelling, and tasting the beer for light-struck flavor aroma.

Synthesis of MBT. To have authentic material for training purposes, MBT was synthesized according to the method of Holscher et al. (*17*). Thiourea (0.4 g) was dissolved, with stirring, in 18% hydrochloric acid (6 mL). Prenol (3-methyl-2-buten-1-ol, 0.25 g) was added, and the solution was allowed to stir for 30 min until it was seen to be homogeneous. The solution was heated to boiling and then allowed to remain stirring overnight at 60 °C. The solution was then made alkaline (pH 11) with potassium hydroxide (30% in water, w/w) and then refluxed for 2 h. The solution was allowed to cool, and *n*-pentane (7 mL) was added; the mixture was shaken vigorously and the pentane layer allowed to separate. The pentane layer, containing the crude MBT as well as some other reaction byproducts, was removed with a glass pipet. No attempts were made to purify the MBT due to the extreme odor involved in working with this compound, even in an efficient fume hood. MBT is stable for several weeks at 4 °C when stored as a dilute solution in dichloromethane (*18*). The pure liquid is less stable and can oxidize and dimerize within 2 weeks at 4 °C (*19*). The MBT was stored as a 10 mg/L aqueous solution at −20 °C.

Determination of Threshold for MBT Solutions. Sensory analysis arguably is the quickest and most sensitive technique for qualitative determination of aroma compounds in beer (*1*). Soltoft (*20*) discussed the advantages of sensory analysis compared with instrument-based methods (mainly speed and sensitivity). The aroma threshold is in the range of 1−30 ng/L (*19*), although some people are considerably more sensitive to this compound than others (*2*).

Thresholds for the synthesized MBT solutions were determined with an expert panel made up of regular expert beer tasters from CUB. Model beer as described above or fresh bitter beer that had been scrupulously shielded from light was dosed with MBT within the range of 1−30 ng/L and presented to the expert panel ($n = 5$). The results, expressed as best estimate thresholds (BET), were calculated for each participant using the equation

$$\mathrm{BET} = (C_m C_n)^{1/2}$$

where C_m is the highest concentration missed and C_n is the next highest (adjacent) concentration.

The group aroma threshold for MBT-dosed model beer was 1 ng/L, and for MBT-dosed bitter lager it was 2 ng/L. These values are within the range of detection obtained previously by the panel. The expert panel was used to assess the aroma of light-struck beers and model beers and agreed that the aroma notes of the model beer and the lager after dosing with MBT were comparable. When model beer and lager were exposed to light, the volatile aromas were comparable, although the model beer had a complexity and a pungency that were slightly different from those of the authentic beer.

Assessment of MBT Formation. Generally, the assessment panel was made up of five trained assessors. The panel assessed the aroma of either model beers or authentic beers, but never compared model beer results with real beer results. Each panelist recorded the intensity of the light-struck character with the following symbols: −, +, ++, and +++, which correspond to no light-struck character, and slight, medium, and strong light-struck character, respectively. Each taster's results were averaged. Goldsmith et al. (*21*) have pointed out the advantages and the drawbacks to using human assessors. It is subjective, and each person has a different threshold; as well, panelists can become saturated. The use of a trained panel and limiting the number of samples assessed in any session as well as averaging the results went some way to addressing these limitations. Thus, the accuracy of the human assessment of the amount of MBT produced was maximized, although it is still an empirical measure. Others have taken the same approach (*16*).

A similar approach was used by Goldsmith et al. (*21*), who evaluated the effect of triplet state quenchers on riboflavin-dependent MBT formation in model beer and in beer. These authors measured second-order rate constants for the quenching of riboflavin fluorescence and the quenching of the riboflavin triplet state by catechin, tryptophan, and ascorbic acid. The effects of the quenchers on light-struck character formation, as judged by sensory panels, in either model beer or regular lager were predicted from the K_q data (*21*), which support the use of this approach.

RESULTS AND DISCUSSION

Spectrophotometric Measurement of Riboflavin Color Loss in Light-Exposed Beer To Determine the Extent of Light-Struck Character Formation. Spectrophotometric analysis of model beer containing 1.2 mg/L riboflavin gave absorbance peaks at 373 and 445 nm which disappeared after a few minutes of exposure of the model beer to light (**Figure 1a−c**). A model beer without riboflavin did not absorb at either of these two wavelengths (**Figure 1d**). According to Heelis (*22*), the light-induced loss of color of riboflavin in solution is due to photochemical reduction of the riboflavin. Riboflavin is excited to a singlet electronic energy state, followed by transition to a triplet energy state, in which it has a strong affinity for electrons and hydrogen ions. The excited riboflavin is able to extract electrons from electron-rich substances such as proteins that may also be in solution (*22*).

Exposure of model beer to light led to a loss of yellow/green color and development of light-struck aroma as confirmed by sensory analysis. If polyphenols such as (+)-catechin or indoles such as tryptophol were added to the model beer before exposure to the fluorescent light, the color loss could be prevented for >1 h and no light-struck character could be detected by sensory analysis. The rate of color loss of riboflavin could be determined by measuring the loss of absorbance at 445 nm (the absorbance peak at 373 nm was found to be smaller and not as clearly defined).

When a model beer was exposed to light, a plot of riboflavin absorbance with time of exposure to the light source produced a curved line, which at first appeared to represent the disappearance of riboflavin from the model beer. However, after a few minutes of exposure to the light source, the clear model beer became cloudy, and this cloudiness interfered with the spectrophotometric determination of riboflavin at 445 nm. Therefore, the rates of disappearance of the absorbance of riboflavin were determined over the first few minutes of exposure of the model beer to light. The cloudiness was

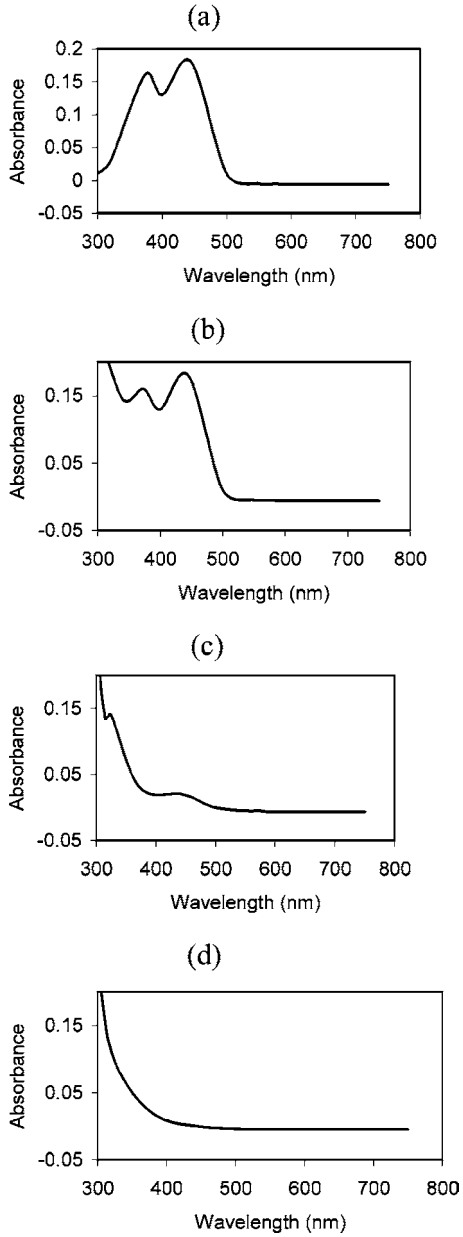

Figure 1. Absorption spectra of (**a**) riboflavin in solution (1.2 mg/L), (**b**) model beer (containing 1.2 mg/L riboflavin), (**c**) light-exposed model beer, and (**d**) model beer without riboflavin.

attributed to a decomposition product of the isohumulones and/or riboflavin.

Linear regression was used to determine the rate of loss of absorbance. Addition of (+)-catechin, (−)-epicatechin, and tryptophol at 10, 50, or 100 mg/L reduced the rate of loss of absorbance of riboflavin in model beer exposed to fluorescent light of intensities 3580 and 4580 lx (**Table 1**). When the riboflavin concentration was increased in the model beer from 1 mg/L (as in real beer) to 10 mg/L to improve the spectrophotometric detection of the riboflavin, (+)-catechin, (−)-epicatechin, and tryptophol still reduced the loss of absorbance and the rate of light-struck aroma formation. When a model beer containing the elevated level of riboflavin was exposed to light, there was a greater difference in the loss of absorbance for riboflavin in the samples treated with (+)-catechin, (−)-epicatechin, or tryptophol compared with the control samples than when a model beer containing 2 or 3 mg/L riboflavin was exposed to the same light intensity. Light-struck character appears when beer is illuminated between 350 and ≈560 nm.

Table 1. Rates of Disappearance of Riboflavin Absorbance (at 445 nm) in a Model Beer Containing 10 mg/L Riboflavin and 10, 50, or 100 mg/L of either (+)-Catechin, (–)-Epicatechin, or Tryptophol[a]

	rate of riboflavin color disappearance (mg L^{-1} min^{-1})		
additive	10 mg L^{-1}	50 mg L^{-1}	100 mg L^{-1}
	Light Intensity of 3580 lx		
no additive	0.36	0.38	0.31
(+)-catechin	0.20	0.12	0.07
(–)-epicatechin	0.20	0.12	0.07
tryptophol	0.18	0.10	0.04
	Light Intensity of 4580 lx		
no additive	0.79	0.75	0.80
(+)-catechin	0.32	0.19	0.07
(–)-epicatechin	0.32	0.19	0.07
tryptophol	0.29	0.17	0.04

[a] Results are an average of five replicate determinations, i.e., $n = 5$.

The effects above are unlikely to be caused by a reduction in light intensity due to the additives themselves. Tryptophol has a molar extinction coefficient of ≈6000 M cm^{-1} at 280 nm, but there is negligible absorbance within the active range of the spectrum between 350 and 550 nm. Likewise, (+)-catechin and (−)-epicatechin have molar extinctions of >25000 at wavelengths of <270 nm, but negligible absorbance in the active spectrum region. The Fe levels in beer are <0.05 ppm [(−)-epicatechin and (+)-catechin > 50 ppm], which avoids the formation of the Fe−tannin complexes, which can absorb significantly in the visible region.

Light exposure of lager containing an additional 0.9 mg/L riboflavin led to light-struck formation as detected by sensory analysis. Addition of (+)-catechin, (−)-epicatechin, or tryptophol at 50 mg/L to the lager prior to light exposure reduced the rate of disappearance of riboflavin (**Figure 2**). The additional riboflavin allowed better determination of riboflavin absorbance because the brown color of beer interfered with the measurement of inherent riboflavin concentration; hence, **Figure 2** shows the disappearance of absorbance of the additional riboflavin. Light-struck character was detected in the dosed beer after 35 min of exposure under the standard conditions. The catechin- and epicatechin-dosed beers were slower to develop the character as shown in **Figure 2** (epicatechin- and catechin-dosed beer curves are superimposed). Tryptophol-dosed beer was slowest to develop the MBT character. The threshold for MBT character in the control beer (plus riboflavin) was ≈35 min; in beer dosed with tryptophol, this was extended to 55 min.

The experiment was repeated with lager containing an additional 1.8 mg/L riboflavin (**Figure 3**) and added polyphenol and tryptophol (50 mg/mL). The effects of (+)-catechin and (−)-epicatechin on the disappearance of riboflavin absorbance from lager were similar. Light-struck character was detected in the lager plus riboflavin control after ≈45 min of exposure to light under the test conditions. This was extended to 60 and 75 min with catechin or epicatechin versus tryptophol, respectively. As found at the lower levels of these compounds, the difference between tryptophol-dosed and the control beer seemed to increase as the light exposure time was increased. For instance, the ++ strength light-struck rating was reached after 80 min for the control, but it took close to 135 min when tryptophol was included. At the end of this experiment, which ran for 200 min, there was no difference in the strength of the light-struck character. The sensory panels agreed that it was overpowering in all cases and at this stage discrimination was impossible.

The rate of disappearance of riboflavin absorbance increased as the concentration of riboflavin in lager increased (**Table 2**).

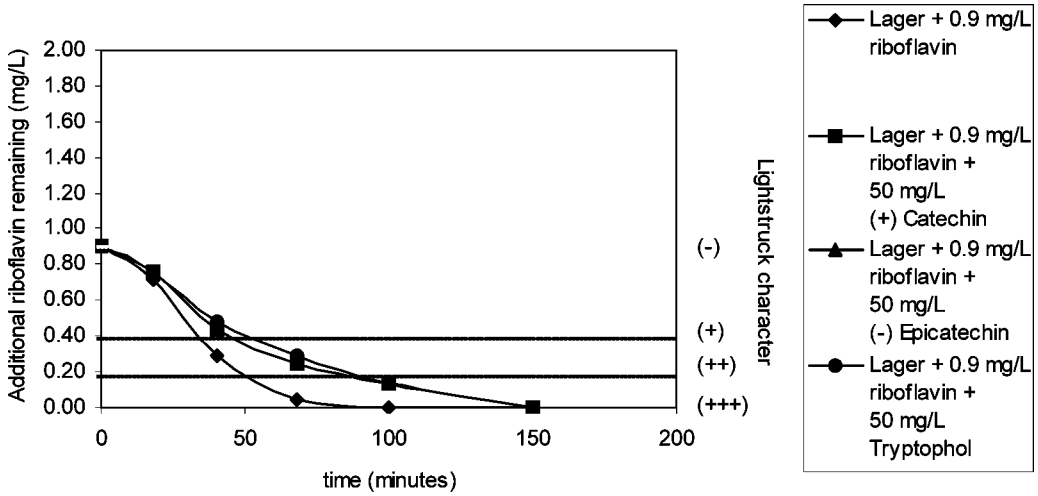

Figure 2. Effect of 50 mg/L (+)-catechin, (–)-epicatechin, or tryptophol addition on the disappearance of absorbance of additional 0.9 mg/L riboflavin from lager (light intensity = 3580 lx). (Each point is an average of five determinations, i.e., $n = 5$.) The curves for samples with added (+)-catechin and (–)-epicatechin are coincident.

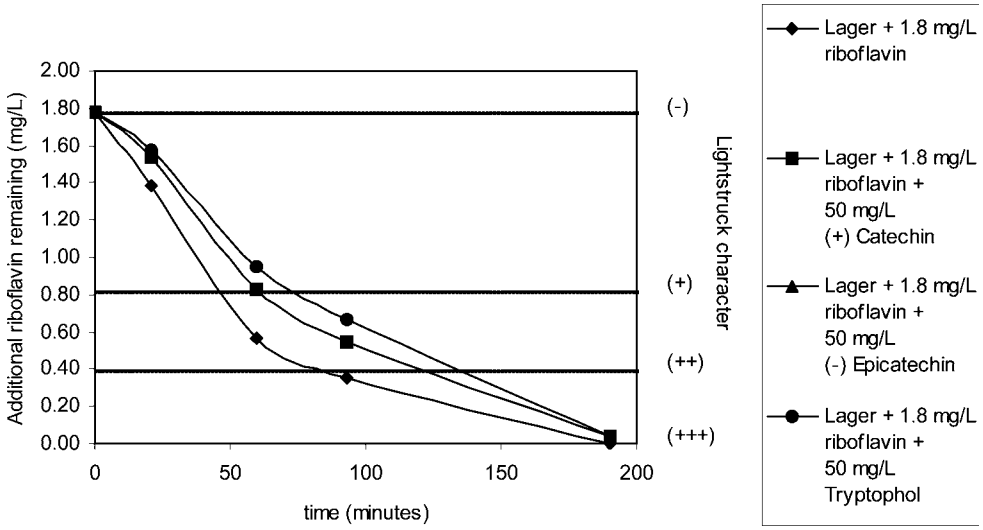

Figure 3. Effect of 50 mg/L (+)-catechin, (–)-epicatechin, and tryptophol addition on the disappearance of absorbance (at 445 nm) of additional 1.8 mg/L riboflavin from lager (light intensity = 3580 lx). (Each point is an average of five determinations, i.e., $n = 5$.) The curves for samples with added (+)-catechin and (–)-epicatechin are coincident.

Table 2. Rates of Disappearance of Additional Riboflavin from Lager Containing (+)-Catechin, (–)-Epicatechin, and Tryptophol[a]

	rate of disappearance of riboflavin absorbance (mg L^{-1} min^{-1})	
additive	0.9 mg L^{-1} riboflavin	1.8 mg L^{-1} riboflavin
no additive	0.15	0.18
(+)-catechin	0.09	0.11
(–)-epicatechin	0.09	0.11
tryptophol	0.07	0.10

[a] Results are an average of five replicate determinations.

Model beers were prepared with 2 and 3 mg/L riboflavin and exposed to light of intensity 3580 lx, but the rate of disappearance of riboflavin absorbance could not be measured because it disappeared too rapidly. This suggests that real beer contains substances that are able to prevent, to some extent, the alteration/degradation of riboflavin.

Effect of Isohumulone, Riboflavin, and Tryptophol on the Disappearance of Riboflavin Absorbance from a Model Beer. To investigate the effect of the concentration of isohumulone and riboflavin on the disappearance of riboflavin absorbance in beer containing the additives (+)-catechin, (−)-epicatechin, or tryptophol, model beers were prepared containing 2, 4, and 6 mg/L riboflavin and various amounts of isohumu-

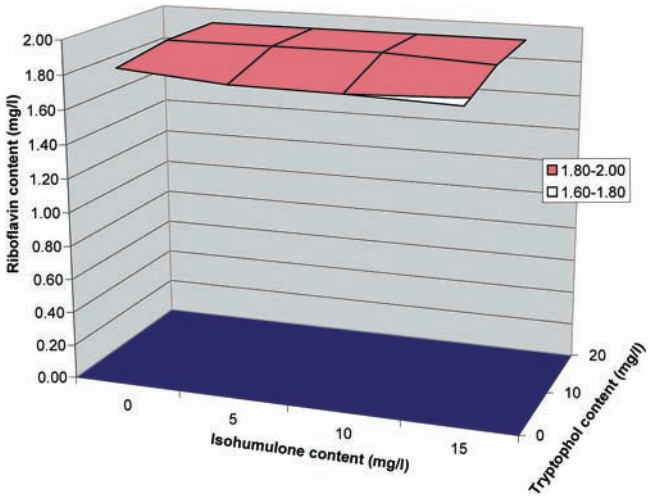

Figure 4. Model beer containing 2 mg/L riboflavin after 10 min of exposure to light of intensity 526 lx.

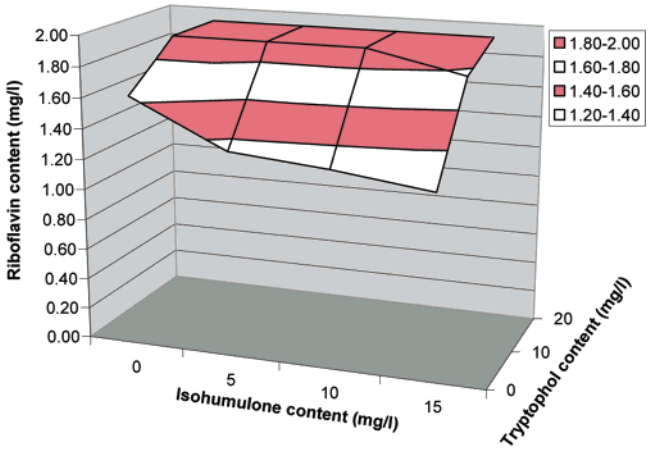

Figure 5. Model beer containing 2 mg/L riboflavin after 31 min of exposure to light of intensity 526 lx.

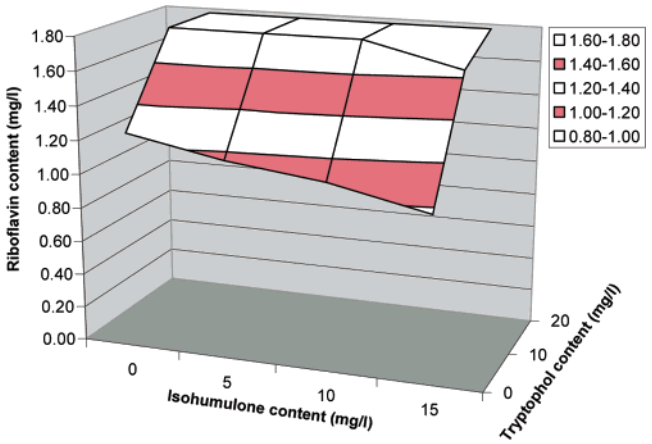

Figure 6. Model beer containing 2 mg/L riboflavin after 60 min of exposure to light of intensity 526 lx.

lones and additives. The model beers were exposed to light of intensity 526 lx, and the disappearance of riboflavin absorbance with time was measured. The low light intensity was chosen to slow the loss of absorbance and so allow data collection. The results of these experiments are summarized in three-dimensional plots that show the effect of time, isohumulone, and either (+)-catechin, (−)-epicatechin, or tryptophol at various concentrations on the disappearance of riboflavin absorbance. Only three-dimensional plots for model beers containing tryptophol are presented here (**Figures 4**−**6**) because all three additives in the model beer led to similar plots. The loss of riboflavin absorbance is represented by pink and white bands in the plots. Each band represents a decrease in riboflavin absorbance of 0.2 mg/L. These plots reveal that riboflavin absorbance disappeared from a solution containing no isohumulones at a markedly lower rate than when isohumulones were present. When isohumulones were added, cloudiness formed in the model beer after 40 min of light exposure, which interfered with the measurement of riboflavin absorbance. The reduced rate of riboflavin disappearance in these model beers was matched by a reduced level of light-struck character, as reported for real beers dosed with polyphenols. The qualitative sensory data show the same trend as the rate of riboflavin change.

The addition of tryptophol before exposure significantly reduced the disappearance of riboflavin absorbance. When indole-2-carboxylic acid was used in place of tryptophol, the result was the same. There was the same reduction in the rate of apparent formation of light-struck character. It was reported by Heelis (*22*) that indoles form complexes with riboflavin in solution. If this happens, indoles may reduce the ability of the riboflavin to interact with isohumulones in real and model beers, but there is no evidence in the literature to suggest complex formation between riboflavin and polyphenols such as (+)-catechin. (+)-Catechin, (−)-epicatechin, and tryptophol were able to preserve riboflavin absorbance in the light-exposed model beer, so there may be another reason for this phenomenon besides complex formation. The polyphenols and indoles cannot donate electrons to riboflavin in the place of the isohumulones because if this occurred, the absorbance at 445 nm would still have disappeared due to formation of photoreduced riboflavin (*22*). However, if riboflavin accepted electrons from the isohumulones and subsequently formed photoreduced riboflavin, the oxidized isohumulones would be electron deficient and unstable (*23*) and possibly may extract electrons from the polyphenols/indoles to regain stability rather than degrade to more stable minor products. The now electron-deficient polyphenols/indoles might then extract electrons from photoreduced riboflavin, possibly returning the riboflavin to its original state before exposure to light. A byproduct of the degradation of isohumulones in beer is MBT, which would otherwise be prevented from forming if the isohumulones did not degrade.

It was discovered during these experiments that when oxygen was bubbled through model beer samples that had lost riboflavin absorbance at 445 nm due to exposure to light, most of the absorbance returned within 1 min which may indicate that the photoreduced riboflavin is oxidized back to the original

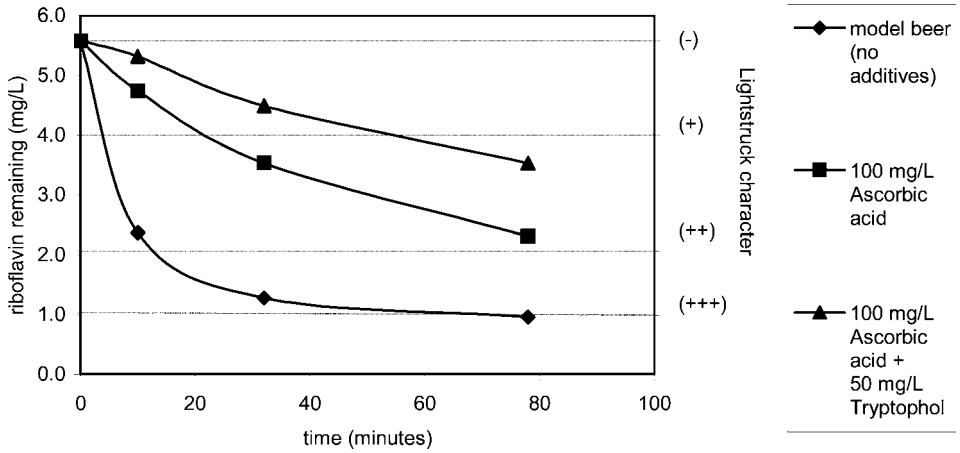

Figure 7. Effect of ascorbic acid plus tryptophol addition on disappearance of riboflavin absorbance from model beer (light intensity = 3580 lx). (Each data point is an average of five determinations, i.e., $n = 5$.)

riboflavin structure or at least to a derivative of riboflavin. Spectrophotometric measurement revealed that 10% of the lost absorbance returned within 5 min after the model beers were removed from exposure to light. Because riboflavin absorbance is maintained during light exposure by the polyphenols and indoles, this suggests that these substances also prevent to some extent the photoreduction of riboflavin. Alternatively, the polyphenols and indoles may quench the excited energy states of riboflavin and prevent formation of the electron-withdrawing triplet state riboflavin. In fact, it was demonstrated recently (*24*, *25*) that the primary photophysical event that leads to light-struck flavor development is the excitation of riboflavin to its triplet state followed by electron transfer from iso-α-acids. Goldsmith et al. (*21*) showed that tryptophan and catechin are able to quench the triplet state and inhibit the formation of light-struck character in beer and in model beers.

Ascorbic Acid as an Inhibitor of Light-Struck Character Formation in Model Beer. Ascorbic acid, a known riboflavin triplet state quencher and antioxidant, was able to diminish light-struck character formation in real and model beers. A model beer containing riboflavin (5.5 mg/L), isohumulones (25 mg/L), and cysteine (20 mg/L) was treated with ascorbic acid (100 mg/L) and tryptophol (50 mg/L) and exposed to light (3580 lx). **Figure 7** shows that the addition of ascorbic acid plus tryptophol prior to exposure of the beer to light was able to decrease the rate of loss of absorbance of riboflavin more effectively than ascorbic acid alone. There was an even greater reduction in the rate of formation of the light-struck character when both were present compared with either individually present. These results were confirmed by sensory analysis. The initial rates of disappearance of riboflavin absorbance from model beer containing ascorbic acid (100 mg/L) and model beer containing tryptophol (50 mg/L) plus ascorbic acid (100 mg/L) were 0.08 and 0.03 mg L^{-1} min^{-1}, respectively.

Thus, by measuring the loss of absorbance at 445 nm, it is possible to model the formation of MBT and to determine the influence of additives such as (+)-catechin, (−)-epicatechin, tryptophol, and ascorbic acid on MBT formation. The relationship between loss of riboflavin absorbance and the subsequent formation of MBT permitted the exploration of the light-struck character in light-exposed beer. We think that this approach can be used by most breweries to evaluate and optimize photostability.

This method showed that in model beer elevated levels of riboflavin led to an increase in the rate of disappearance of absorbance. However, lager containing similarly elevated levels of riboflavin lost riboflavin absorbance at a lower rate than model beers, suggesting that substances in lager may prevent to some extent the photodegradation of riboflavin. Beer is complex in composition. There are other small compounds in beer apart from the ones tested above that may affect riboflavin function. Riboflavin may also bind to macromolecules and avoid activation. As mentioned tryptophan, and some indoles, at similar concentrations in beer and model beer will protect riboflavin from photodegradation. There are many process variations in beermaking, different hopping procedures, different hop varieties, and different malts and yeast in use. Commercially this should improve the chances of suppressing MBT formation, without ever contravening the fastidious German "rheinheitsgebot" beermaking law.

Addition of (+)-catechin, (−)-epicatechin, tryptophol, and ascorbic acid would markedly reduce but not prevent light-struck character formation in lager. The effects of (+)-catechin and (−)-epicatechin on the disappearance of riboflavin absorbance were similar. The addition of ascorbic acid plus tryptophol prior to exposure of beer to light was able to decrease the rate of loss of riboflavin absorbance more effectively than the addition of ascorbic acid alone.

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