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Polysaccharides influence on the interaction between tannic acid and haze active proteins in beer

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ABSTRACT

Colloidal instability in beer is mainly caused by interactions between proteins and polyphenols. These two combine producing a visible haze that reduces the physical shelf life of beer. The haze active proteins (HAPs) react with tannic acid (TA); therefore, this reaction provides a way to determine HAP concentrations in beer. Beers also contain a number of constituents that may influence the protein–polyphenol haze formation. We used a response surface methodology to predict the influence of total polysaccharides (TPS) and proteins on beer haze. Experiments were carried out using the Central Composite Design (CCD) methodology. Samples of beer were prepared with variable concentrations of TPS and proteins. TPS concentrations ranged between 1.34 and 2.23 g L^{-1} and proteins concentrations between 0.11 and 0.18 g L^{-1}. Results show that the increase in turbidity in response to TPS addition was similar to that in response to protein addition. Our regression analyses indicate a significant dependency and correlation between the observed values and the predicted response values (R^2 = 97.87% and R^2[adj] = 95.75%). Furthermore, these values indicate that our experimental model can explain 95.75% of the total variation. Therefore, using TA as an indicator of the interaction of TPS with proteins, as commonly done, can lead to considerable errors, since the polysaccharides also react with TA, and this reaction actually causes a considerable increase in turbidity.

Introduction

Beer after fermentation shows significant turbidity due to the presence of yeast and the cellular tissue comminuted during the malt processing (Benítez, Martinez Amezaga, Sosa, Peruchena, & Lozano, 2013; Fleet & Siebert, 2005; Siebert, 2006; Van der Sman, Vollebregta, Mepschen, & Noordman, 2012). To produce a clear beverage with good visual appearance the colloidal particles must be removed (Fleet & Siebert, 2005; Siebert, 2006; Steiner, Becker, & Gastl, 2010). Beer is typically filtered with diatomaceous earth (Atkinson, 2005; Bamforth, 2009). Subsequently, the stabilization stage continues to prevent the formation of postpackaging haze (Bamforth, 2009; Briggs, Boulton, Brookes, & Stevens, 2004).

The most common cause of postpackaging haze is due to the interaction between proteins and polyphenols, eventually producing variably-sized colloidal particles (Siebert, 2009; Steiner et al., 2010).

Two fundamental types of proteins were identified in beer: those that cause foam, which must be retained, and those responsible for haze formation, which should be eliminated. A number of approaches have been used to determine the amount of HA-proteins in samples. The most successful approach consists of adding a fixed amount of TA to a sample (Bamforth, 2009; Siebert, 2006, 2009). After incubating the sample, any measured increase in turbidity is expected to be proportional to the amount of HA-proteins in the sample. The advantage of this approach is that only substances that are able to form haze with polyphenols will respond (Siebert, 2006, 2009).

Beers also contain a number of constituents, such as alcohol and the hydrogen ion (pH), which can influence the protein–polyphenol haze formation. In this paper, two other factors are studied: ionic strength and polysaccharides concentration.

The ionic strength can modify the turbidity response of the interaction of proteins with TA. Benitez, Genovese, and Lozano (2007a) studied the effect of KCl on the colloidal particles of apple juice. This salt modified the electric potential of the colloidal particles but not its stability. Therefore, as the behavior of the interaction of TA with apple juice is similar to that of TA with beer, KCl was used in this study. Finally, the most inorganic salts usually found in beer are potassium (200–

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450 mg L^{-1}), as cations, and chloride (120–500 mg L^{-1}) and phosphate (170–600 mg L^{-1}), as anions (Buiatti, 2009).

Gelatin has the ability to bind to TA in the same way as do beer proteins, because of their similar composition in proline and degree of denaturation. Oh, Hoff, Armstrong, and Haff (1980) showed that ionic strength influenced the hydrophobic bonding between TA and either gelatin or poly-proline. Different interactions of gelatin and beer proteins, with TA could indicate differences in the composition of the liquid medium surrounding the proteins and, thus, differences in the influence of the ionic strength.

Some researchers argue that haze can be stabilized by reducing either only proteins or only polyphenols, because according to them, polysaccharides do not participate in the mechanism that forms haze, but are instead simply incorporated as haze particles (McMurrough, Madigan, Kelly, & O'Rourke, 1999; Siebert, 1999, 2006, 2009).

Proteins react with TA, making TA useful to determine protein concentrations in beer. To predict the influence of total polysaccharides (TPS) and proteins on beer haze we used a response surface methodology. In addition, we investigated the effect of ionic strength on the nephelometric turbidity response (τ) during haze formation. Finally, we propose an experimental model to predict the influence of TPS and proteins on TA turbidity (τ_{TA}).

Materials and methods

Beer preparation

Mashing was carried out in a 40 L stainless steel container. The procedure started by mixing 7.5 kg of barley malt from Argentina (Cargill Malt Division) with deionized water at 62 °C for 90 min. Subsequently, the wort was boiled for 1 h with the addition of hops for bitterness and flavor. This bitter wort was left to settle for 30 min before it was cooled to 12 °C. The wort was pitched with Lager yeast (Saflager S-23, Fermentis, France). The fermentation was carried out at 12 °C for a period of 15 days, followed by a maturation period of 7 days and a cold rest at 3 °C for another 2 days. Finally, each prepared samples was filtrated using a Buchner funnel (Ø = 50 mm) with a filter bed consisting of a pre-coat of 1 g diatomaceous earth (Standard Super-Cel, mean porosity = 3.5 μm, permeability = 2.8×10^{-13} m^2, Refil, Argentina) over a filter paper Whatman No. 3 under vacuum (−50 kPa). All samples were prepared in triplicate (Benítez et al., 2013).

Colloidal particles determination

Turbidity depends on the concentration, size, and relative refractive index of its particles in suspension (McClements, 2005). The average diameter ($\overline{D}$) and the nephelometric turbidity (τ) are easy to measure and are widely used as parameters to describe particle size and turbidity of commercial beverages. The expression of Eq. (1) was used to describe the turbidimetric behavior of colloidal particles in apple juice (Benitez, Genovese, & Lozano, 2007b; Benitez, Lozano, & Genovese, 2010) and beers (Benítez et al., 2013) and derives from the following equation proposed by Dobbins and Jizmagian (1966):

$$\tau = \frac{3}{2}\frac{C}{\rho_m}\frac{Q_{av}}{\overline{D}} \quad (1)$$

where ρ_m is the density of the continuous phase, C is the particle concentration, and Q_{av} is the nephelometric average scattering efficiency of the polydispersed and irregular particles.

As predicted by Eq. (1), beer turbidity before filtration was found to be directly proportional to particle concentration. Consequently, experimental data were fitted with straight lines through the origin:

$$\tau = \tau_e C \quad (2)$$

where τ_e is the specific turbidity (Dickinson, 1994).

In the present work, Eq. (2) is used to predict the concentration of the initial colloidal particles of beer (C_0).

Ionic strength modification

The influence of the ionic strength on the interaction of proteins and TA was evaluated with two assays. The first assay consisted of mixing the beer samples with increasing additions of KCl (0.02, 0.04, 0.06, 0.08 and 0.1 M) and a subsequent addition of a solution with a constant concentration of TA (Sigma-Aldrich, Germany). Turbidity was measured after 30 min. The second assay consisted of using a gelatin (Sigma-Aldrich, Germany) solution with the same concentration as proteins in the beer samples. The alcohol level was fixed with ethanol 96% (Biopack, Argentina) and the pH = 4 was fixed with a buffer solution (Biopack, Argentina). The gelatin solution was added with increasing concentration of KCl (0.02, 0.04, 0.06, 0.08 and 0.1 M) and a subsequent addition of a solution with a constant concentration of TA. Turbidity was measured after 30 min. Due to the difference in ionic strength between the beer sample and gelatin, the electrical conductivity of the beer sample was measured and correlated with the ionic strength of a KCl solution (Benitez et al., 2007a). This correlation provides an estimate for the ionic strength of the beer sample.

Polysaccharides interference

Preparation of a TPS concentrated solution

HA-proteins from beer were extracted with bentonite (0.5 wt.%/volume commercial sodium bentonite type I; La Elcha; Mendoza, Argentina) (Benítez & Lozano, 2007) and polyvinylpolypyrrolidone (15 g L^{-1}, Polyclar 10, International Specialty Products, Argentina) (Mitchell, Hong, May, Wright, & Bamforth, 2005). Bentonite is used for protein removal (Sadosky et al., 2002; Siebert, 2009). The negative reaction resulting from the Bradford method (Bradford, 1976) for proteins and the Folin–Ciocalteu method for total polyphenols (Singleton, Orthofer, & Lamuela-Raventos, 1999) was used to verify whether haze active precursors had been removed. TPS were extracted by ethanol (80%) precipitation and drying at 40 °C, as described by Segarra, Lao, López-Tamames, and De la Torre-Boronat (1995).

Assay to determine the interaction of TPS on TA turbidity (τ_{TA})

It is well known that TA selectively combines with HA-proteins, but it is unknown whether it interacts with TPS. To explore this interaction TPS solution with increasing concentrations, range from 0.2 ± 0.05 to 3.2 ± 0.4 g L^{-1}, were added to five samples of beer and water, respectively. The solutions were left to settle for 30 min, and turbidity was measured. Subsequently, a TA solution (55 mg L^{-1}) was added to the samples. The solution was left to settle for another 30 min, and turbidity was measured again.

Assay to determine the interaction of TPS on gelatin turbidity (τ_G)

It is well known that gelatin selectively reacts with polyphenols forming haze (Benítez & Lozano, 2007; Siebert, 2006, 2009), but gelatin–polysaccharides interactions are poorly understood. Therefore, TPS solutions with increasing concentrations, range from 0.2 ± 0.05 to 3.2 ± 0.4 g L^{-1}, were added to water and beer samples, respectively. Turbidity was measured after 30 min, and then a gelatin (Sigma-Aldrich, Germany) solution (55 mg L^{-1}) was added (Van Buren, 1989). Solutions were left to settle for 30 min, and turbidity was measured again.

Assay to determine the interaction of TPS and HA-proteins on τ_{TA} and τ_G

The response surface methodology (RSM) was used to study the simultaneous influence of TPS and HA-proteins on τ_{TA}, as it allows us to find the optimal variation and indentify the influence of both factors. The 3D response surface was used to determine the individual and cumulative effect of the factors and the mutual interaction between the factors and the dependent variable.

Beer proteins were obtained by concentrating the supernatant, without bentonite treatment, after TPS and polyphenols extraction. Supernatant concentration was done under vacuum without exceeding the 60 °C in order to eliminate the ethanol used for TPS extraction. Considering that both alcohol and pH could modify τ_{TA}, samples were set to 4% ethanol and pH = 4 (Biopack, Argentina). TA was added to the samples, and turbidity was measured after 30 min. Gelatin was added after 24 h, and turbidity measured again (τ_G). A response surface analysis was used to characterize the effects of TA and gelatin. Each factor was set at two different levels (k). The total number of experimental runs (N) was calculated as:

$$N = 2^k + 2 \cdot k + x_0 \quad (3)$$

were $x_0 = 3$ is the number of central points. Least squares regression methodology was used to obtain estimates for the parameters (Kumar, Prasad, & Mishra, 2008):

$$\tau = \beta_0 + \sum \beta_i X_i + \sum \beta_{ii} X_i^2 + \sum \beta_{ij} X_i X_j + \varepsilon \quad (4)$$

where β_0 is the constant, β_i is the slope or linear effect of the input factor X_i, β_{ii} is the quadratic effect of the input factor X_i, β_{ij} is the interaction effect between the input factors x_i, and ε is the residual error.

The experiments were carried out randomly using the Central Composite Design (CCD) methodology. The resulting responses for each combination of variables are listed in Tables 2a and 3a.

Measures

Protein content was determined using the Bradford method (1976). Total polyphenol (TPP) content and TPS content were determined with the Folin–Ciocalteu method (Singleton et al., 1999) and the Phenol–Sulfuric method (Segarra et al., 1995), respectively.

Turbidity (τ) was measured at 20 °C in a 15 mL standard vial using a PC Compact Nephelometer (Oaklon T-100, USA). Each sample was measured in triplicate and left to settle in the vial for 30 min before measurements.

Sample preparation for scanning electron microscopy

For scanning electron microscopy (SEM) analyses of the particles, a sample of diluted beer before filtration (1 mL of beer in 100 mL of water) and a sample of non-diluted beer after filtration were fixed with 2.5% glutaraldehyde in phosphate buffer with pH = 7.2. Dilution of the beer before filtration is needed to reach a similar density of particles as in the non-diluted beer after filtration. Droplets of each fixed sample were put on glass coverslips with polylysine film for 1 h. Polylysine (ε-poly-L-lysine, EPL) is a polymeric substance with a net electrostatic potential that attracts particles (Thomas, Coakley, & Winters, 1996). The coverslips were then washed with phosphate buffer, dehydrated with 25%, 50%, 75%, and 80%, and three times 100% acetone solutions, and, finally, desiccated at critical dry point (Polaron E3000 CPD, U.S.A.) with acetone and CO_2 as intermediate fluids. The samples were covered with gold using an automatic sputter coater (Sputter Coater, Pelco 91000) and analyzed by SEM (LEO, EVO 40, Cambridge, Eng.) at 10 kV accelerating voltage.

Statistical analyses

Data points were presented as the mean of the measured values. The variance was analyzed, and the Turkey test was performed at the 0.05 level of significance.

The statistical software package MINITAB® Release 15 Statistical Software for Windows et al. (2006) (Minitab Inc., USA) was used for regression analysis and for estimation of coefficients of the regression equations.

Results and discussion

Ionic strength modification

It is well known that the isoelectric point of proteins is affected by pH and ionic strength, and changes in the ionic strength of beer could affect τ_{TA}. For this reason, the ionic strength of beer samples was estimated using KCl to modify it and to correlate beer conductivity with KCl solution conductivity.

Beer electrical conductivity was 3.6 ± 0.3 mS, implying an ionic strength of 0.05 ± 0.01 M, based on KCl concentration. The selected average concentration of beer proteins was 0.08 ± 0.03 g L^{-1}, obtained by mixing the beer with a protein-free solution at initial pH, alcohol degree, and ionic strength.

Fig. 1 shows the effect of KCl on τ_{TA}. Turbidity remained stable at any ionic strength after adding TA, whereas it was effectively reduced by gelatin with increasing additions of KCl. It was presumed that beer components act as a buffer with respect to changes in ionic strength. This buffer effect was not observed when gelatin was added. Therefore, when gelatin or any other clarifying protein is used with the tannic acid test, alcohol content, pH, and ionic strength should be controlled. However, when tannic acid is used to form beer turbidity, it is not necessary to control ionic strength.

Total polysaccharides (TPS)–beer natural protein (P) interaction

As in previous studies (Benítez et al., 2013; Siebert, 2006; Siebert, Carrasco, & Lynn, 1996), to measure turbidity of beer, samples were diluted to fit the range of values effectively measured by the turbidimeter. The initial protein concentration was 0.18 ± 0.03 g L^{-1}.

In both water and beer, turbidity increased linearly after adding TPS (Fig. 2), but starting from a different initial turbidity. Compared to water, filtered commercial beer has a residual turbidity ($\tau_0 = 2$ NTU). Therefore, a more appropriate equation to describe beer turbidity after filtration would be:

$$\tau = \tau_0 \tau_e \cdot C. \quad (5)$$

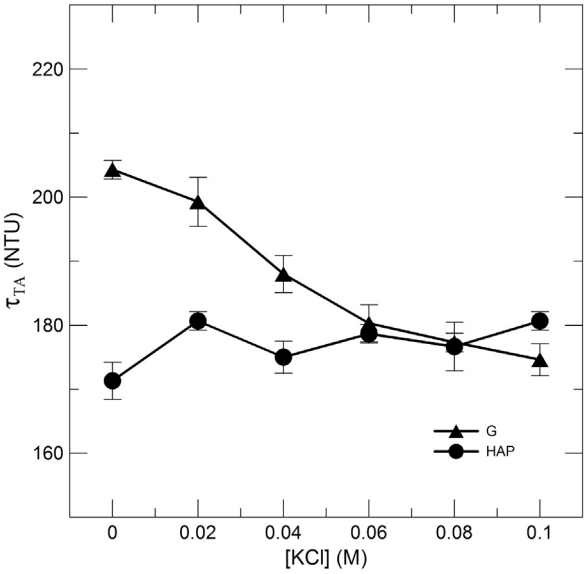

Fig. 1. Effect on τ_{TA} of beer HA-proteins and gelatin solution with different ionic strength.

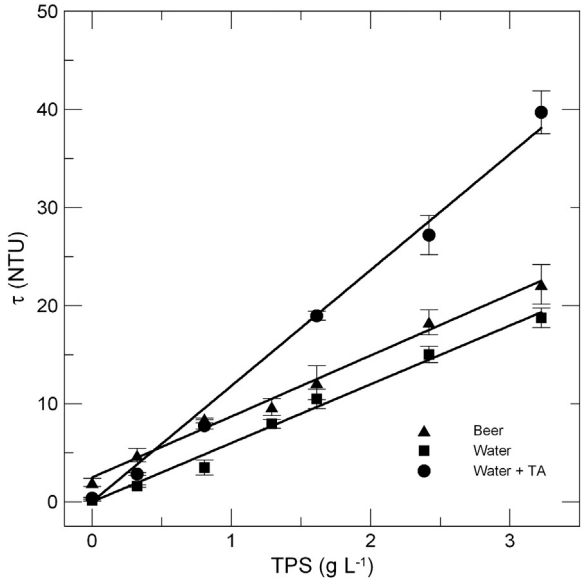

Fig. 2. Effect on τ of TPS addition to samples of beer, water, and water with a later addition of tannic acid.

Residual turbidity can be attributed to colloidal particles, as those identified by our SEM analyses (Fig. 3b) and present also before beer filtration (Fig. 3a). As reported in previous work (Benítez et al., 2013), the average size of these particles is 0.06 μm.

From Eq. (1), the calculated initial concentration of particles is $C_0 = 5.15 \pm 0.01$ g L^{-1}, with $Q_{av} = 0.35$ NTU·m and $\rho_m = 1.008 \pm 0.001$ g mL^{-1} (Benítez et al., 2013). Colloidal particles not retained by the filter consist mostly of polysaccharides, representing 97%, and only minimally of proteins, representing only about 2% and polyphenols, less than 0.3% (Benítez et al., 2013). Note also, that the concentration of TPS added to the beer samples was lower than the initial concentration of colloidal particles also consisting mainly of polysaccharides.

As listed in Table 1, the specific turbidity (τ_e) for water and beer samples with increasing addition of polysaccharides had statistically similar results ($p < 0.05$). In the case of water, the increase in turbidity was linear, although the turbidity response was low. The difference for the solution with the highest polysaccharides concentration in water, before and after adding TA, was of 21 ± 1 NTU. In all analyzed cases, Eq. (5) was acceptable, since the data correlated with a linear adjustment (Fig. 4).

A significant effect occurred when TA was added to the beer samples. Considering that both HA-proteins and HA-polyphenols were removed before the addition of TA in water samples, the linear increase in turbidity can be attributed to the interaction of the TPS with TA. In the case of the beer–TPS samples, TA produced a significant increase in turbidity, similar to that found by Siebert (2006, 2009). This increase is due to the interaction between proteins and polyphenols, with a maximum τ_{TA} at a specific TPS concentration. Polysaccharides and beer proteins has been thought to organize in a matrix, which significantly increases turbidity if combined to TA. This holds until the maximum turbidity value is reached, after which the activated proteins–polyphenols' sites saturate, and turbidity diminishes (Siebert, 2009).

Results indicated that the contribution of polysaccharides to turbidity is actually higher than the contribution of alcohol and pH, as found also by Siebert (2006, 2009), and should thus not be ignored. Moreover, previous work (Benítez et al., 2013) found that only 20% of TPS were retained by conventional filtration. It was assumed that, in this case, an increase in size of the colloidal aggregates, not contemplated by Eq. (1), could substantially modify Q_{av}. Therefore, another methodology needed to be considered to identify HA-protein and TPS influence on turbidity.

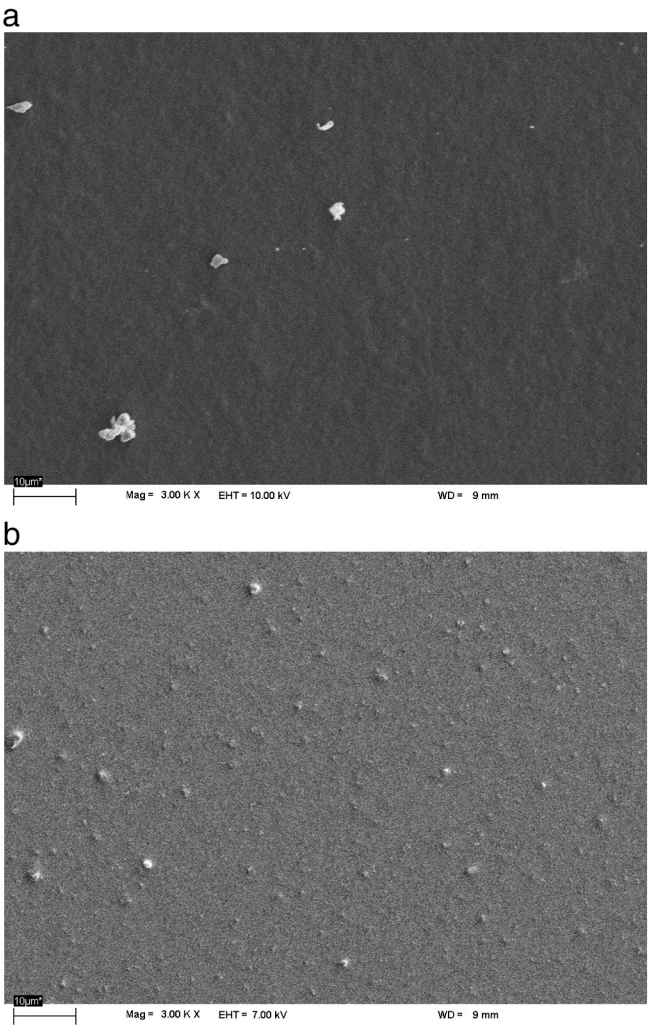

Fig. 3. SEM micrograph of particles of: a) a diluted sample of beer (1 mL of beer in 100 mL of water) before filtration and b) a non-diluted sample of beer after filtration. Magnification: 3000×. Scale bar: 10 μm = 100 pixels.

Total polysaccharides (TPS)–beer natural polyphenols (PP) interaction

The effect on turbidity of gelatin addition in beer and in water was smaller than that of the TA addition (Fig. 5). However, the specific turbidity increased more with gelatin in water than with gelatin in beer (Table 1). This result seems to indicate that polysaccharides added in beer interacted with the natural proteins of beer, blocking the access of gelatin to active sites and, consequently, reducing the possibility for gelatin to interact with proteins and contribute to turbidity. Therefore, interaction between polysaccharides and proteins was confirmed in this assay.

Table 1
Setting parameters for Eq. (5). Samples with addition of TPS. TA and gelatin treatment.

	τ_e (NTU L g^{-1})	τ_0 (NTU)	R^2
Water	0.60 ± 0.05a	0 ± 0.05a	0.989
Water + TA	1.16 ± 0.04b	0 ± 0.05a	0.985
Beer	0.63 ± 0.05a	2.4 ± 0.2b	0.992
Water + gelatin	0.94 ± 0.04c	0 ± 0.04a	0.981
Beer + gelatin	0.74 ± 0.06d	2.4 ± 0.03b	0.991

τ_e (N = 5) and τ_0 (N = 5) data are mean values ± standard deviation. Means in same column with different lowercase letters are significantly different ($p < 0.05$).

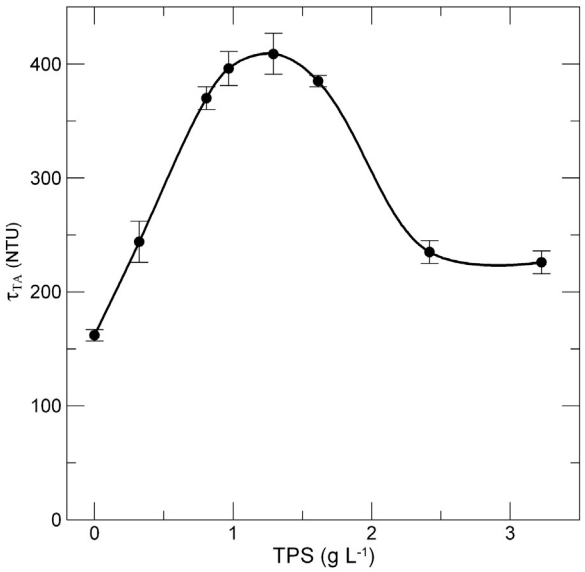

Fig. 4. Effect on τ_{TA} of TPS addition to beer samples.

Gelatin was added to identify the effect of TPS on polyphenols. However, turbidity was lower in beer than in water. Siebert (2009) considered that polyphenols would polymerize before they would interact with polysaccharides. Hence, it may be assumed that no polymerized polyphenols are found in beer after filtration (Benítez et al., 2013).

Interaction of the HA-proteins and TPS on the turbidity response of tannic acid (τ_{TA})

Table 2a lists the matrix of TPS and proteins obtained with the CCD methodology, and the experimental τ_{TA} value after the interaction with TA. Results show that, with constant protein content (at 0.1438 g L^{-1}), polyphenols reached a maximum concentration of 1.34–2.23 g L^{-1}. Beer turbidity significantly varies under these experimental conditions. Fig. 6 also shows a maximum for both proteins and TPS variables. However, the maximum was higher in the case of proteins, as shown by the response surface of τ_G versus TPS and proteins. We obtained significant estimated values of linear regression coefficients for TPS ($p = 0.046$) and proteins ($p = 0.000$), and significant

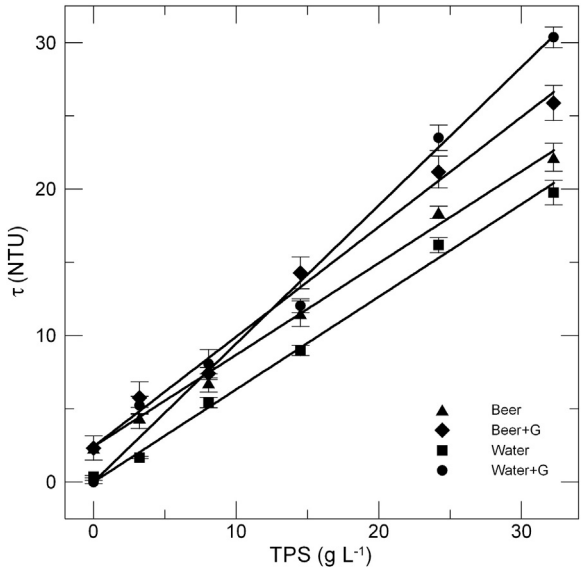

Fig. 5. Effect on τ of TPS addition to water and beer samples with a later addition of gelatin.

Table 2a
CCD matrix of TPS and P along with experimental τ value after the interaction with TA.

Std. order	Run order	Pt type	Blocks	TPS (g L^{-1})	P (g L^{-1})	τ_{TA} (NTU)
4	1	1	1	2.2325	0.1798	496.0
8	2	−1	1	1.7860	0.1798	509.3
3	3	1	1	1.3395	0.1798	495.3
2	4	1	1	2.2325	0.1079	467.6
10	5	0	1	1.7860	0.1438	576.7
5	6	−1	1	1.3395	0.1438	555.7
9	7	0	1	1.7860	0.1438	576.7
7	8	−1	1	1.7860	0.1079	487.3
1	9	1	1	1.3395	0.1079	497.2
11	10	0	1	1.7860	0.1438	576.7
6	11	−1	1	2.2325	0.1438	534.7

Central composite design: factors, 2; base runs, 11; base blocks, 1; replicates, 1; total runs, 11; total blocks, 1. Two-level factorial: full factorial: cube points, 4; center points in cube, 3; axial points, 4; center points in axial, 0. The analysis was done using coded units.

quadratic regression coefficients for TPS ($p = 0.013$) and proteins ($p = 0.000$). Significant squared terms indicate that a curved line relationship exists between turbidity and the square factors. However, the quadratic regression coefficient for the interaction between TPS and proteins was not significant ($p = 0.131$) (Table 2b).

A positive sign of the coefficient represents a synergistic effect, whereas a negative sign indicates an antagonistic effect. The positive linear regression coefficients indicate that an increase of these factors goes along with an increase of τ. However, the negative quadratic regression coefficients are larger, indicating that, in the studied zone, both factors have an antagonistic effect causing a reduction of τ after the maximum peak.

A regression equation using the coefficients is represented as follows:

$$\tau_{TA} = -689.9 + 268 \cdot TPS + 14,222 \cdot P - 99.4 \cdot TPS^2 - 51,590.4 \cdot P^2 + 471.3 \cdot TPS \cdot P \quad (6)$$

where τ_{TA} is the response for the interaction of the variables with TA. The low standard deviation value (8.380) between the measurements and the estimates indicates that the equation appropriately represents the relationship between the response and the significant variables.

The high values of R^2 (97.87%) and R^2 (adj) (95.75%) indicate a high dependency and a correlation between the observed values and the predicted response values. Furthermore, these values indicate that this model can explain 95.75% of the total variation.

The statistical significance of the ratio of the mean square variation due to regression and mean square residual error was tested using analysis of variance (ANOVA). F values for all regressions were high (46.01,

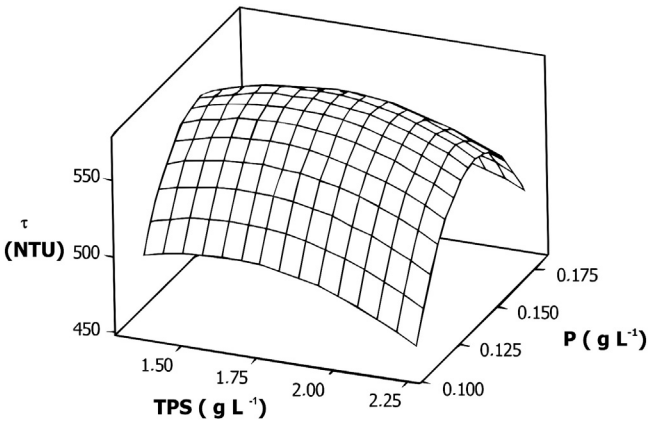

Fig. 6. Response surface of τ versus TPS and proteins. Haze-forming agent: tannic acid. Variables coded in Table 2a.

Table 2b
Estimated regression coefficients for the variables TPS and proteins (P) on τ_{TA}.

Term	Coefficient	S.E. coefficient	T	p
Constant	−689.9	120.8	−5712.0	0.002
TPS (g/L)	268	101.8	2636.0	0.046
P (g/L)	14,222	1264.6	11,247.0	0.000
TPS (g/L) × TPS (g/L)	−99.4	26.4	−3762.0	0.013
P (g/L) × P (g/L)	−51,590.4	4073.3	−12,665.0	0.000
TPS (g/L) × P (g/L)	471.3	261.0	1806.0	0.131

S = 8.380, PRESS = 2513.64, R^2 = 97.87%, R^2 (pred) = 84.77%, R^2 (adj) = 95.75%.

Table 2c
Analysis of variance for % τ_{TA} versus TPS (g L^{-1}), proteins (g L^{-1}) in coded units.

Source	DF	Seq. SS	Adj. SS	Adj. MS	F	p
Regression	5	16,157.80	16,157.80	3231.55	46.01	0.000
Linear	2	807.60	9845.20	4922.59	70.08	0.000
Square	2	15,121.10	15,121.10	7560.57	107.64	0.000
Interaction	1	229.00	229.00	229.02	3.26	0.131
Residual error	5	351.20	351.20	70.24		
Lack-of-fit	3	351.20	351.20	117.06		
Pure error	2	0.00	0.00	0.00		
Total	10	16,509.00				

see Table 2c, vs $F_{0.05;\ 5,5} = 5.05$), according to the F-distribution tables for $F_{0.05}$ (Montgomery, 2003), indicating that most of the variation in the response can be explained by the regression equation. Based on the ANOVA analyses, the coefficients for the linear and square terms were significant (p = 0.000 in both cases), whereas the interaction effect was not significant (p = 0.131). Therefore, the prediction model works. Because no residual error was found (see Table 2c), means that the model effectively explains the variation in the response data.

Considering the form of the surface and the fact that a decrease is due to a saturation of the active sites, it would be convenient to use the increasing zone of the curve to infer the real concentration of HA-proteins based on turbidity and TPS. In this way, it is possible to evaluate the quantity of stabilizing agents needed to be added.

Interaction of the HA-proteins and TPS on the turbidity response of gelatin

The gelatin experiment was also used to evaluate the saturation of active sites by TA aggregation to the matrix of TPS and proteins. Samples were left to interact with TA for 24 h, and then a gelatin solution was added. The maximal depression observed in Fig. 7 corresponds with the peak observed in Fig. 6, even though there is a larger variation due to the TPS rather than the proteins. This could be explained by the fact that the TPS interact with both the proteins and the TA, and, therefore, the variation of τ_G is larger (Table 3a).

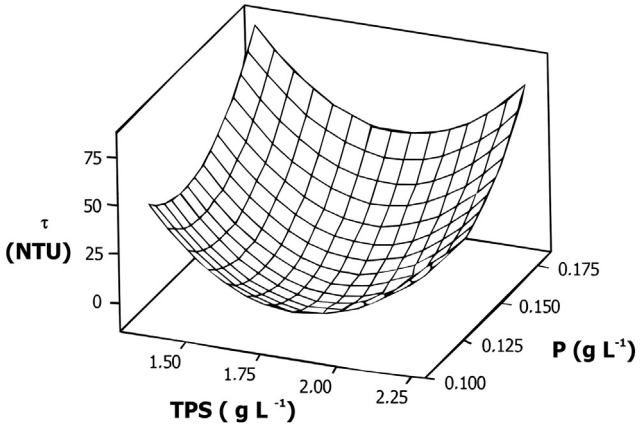

Fig. 7. Response surface of τ_G versus TPS and P. Variables coded in Table 3a.

Table 3a
CCD matrix of TPS and P along with experimental τ_G.

Std. order	Run order	Pt type	Blocks	TPS (g L^{-1})	P (g L^{-1})	τ_G (NTU)
1	1	1	1	1.3395	0.1079	45.3
8	2	−1	1	1.7860	0.1798	16.3
11	3	0	1	1.7860	0.1438	0.0
6	4	−1	1	2.2325	0.1438	21.0
7	5	−1	1	1.7860	0.1079	2.3
2	6	1	1	2.2325	0.1079	33.3
4	7	1	1	2.2325	0.1798	82.7
9	8	0	1	1.7860	0.1438	0.0
5	9	−1	1	1.3395	0.1438	24.7
3	10	1	1	1.3395	0.1798	94.7
10	11	0	1	1.7860	0.1438	0.0

Central composite design: factors, 2; base runs, 11; base blocks, 1; replicates, 1; total runs, 11; total blocks, 1. Two-level factorial: full factorial: cube points, 4; center points in cube, 3; axial points, 4; center points in axial, 0. The analysis was done using coded units.

Significant estimated values of linear regression coefficients were obtained for TPS (p = 0.010) but not for proteins (p = 0.063). On the other hand, quadratic regression coefficients for TPS (p = 0.008) and proteins (p = 0.037) were significant in both cases. However, the quadratic regression coefficient for the interaction between TPS and proteins was not significant (p = 1.000). It can be observed that a positive constant and negative linear regression coefficients for both variables were obtained, suggesting an antagonistic effect on τ_G. The largely positive quadratic regression coefficients indicate a synergetic effect in the studied zone, resulting in an increase of τ_G after the maximum peak. A regression equation based on the studied coefficients is represented as follows:

$$\tau_G = 987.3 - 719.6 \cdot TPS - 5279 \cdot P + 198.6 \cdot TPS^2 + 20165.3 \cdot P^2 - 0.1 \cdot TPS \cdot P. \quad (7)$$

The high values of R^2 (90.15%) and R^2 (adj) (80.30%) indicate a high dependency and a correlation between the observed values and the predicted response values (Table 3b).

All F-values were high (9.15), according to the F-distribution tables for $F_{0.05}$ (Montgomery, 2003), indicating that the variation in the response can largely be explained by the regression equation. According to the ANOVA analyses, both coefficients for the linear terms and the square terms were significant (p = 0.012 and p = 0.005, respectively)

Table 3b
Estimated regression coefficients for the variables TPS and P on τ_G.

Term	Coefficient	S.E. coefficient	T	p
Constant	987.3	211.82	4.661	0.006
TPS (g/L)	−719.6	178.52	−4.031	0.010
P (g/L)	−5279	2217.90	−2.380	0.063
TPS (g/L) × TPS (g/L)	198.6	46.30	4.289	0.008
P (g/L) × P (g/L)	20,165.3	7142.43	2.823	0.037
TPS (g/L) × P (g/L)	−0.1	457.66	0.000	1.000

S = 124.6923, PRESS = 8973.61, R^2 = 90.15%, R^2 (pred) = 81.09%, R^2 (adj) = 80.30%.

Table 3c
Analysis of variance for % τ_G versus TPS (g L^{-1}) and proteins (g L^{-1}) in coded units.

Source	DF	Seq. SS	Adj. SS	Adj. MS	F	p
Regression	5	9875.64	9875.64	1975.13	9.15	0.015
Linear	2	5160.51	5160.51	2580.25	11.95	0.012
Square	2	7627.21	7627.21	3813.61	17.67	0.005
Interaction	1	0.00	0.00	0.00	0.00	1
Residual error	5	1079.30	1079.31	215.86		
Lack-of-fit	3	1079.30	1079.31	359.77		
Pure error	2	0.00	0.00	0.00		
Total	10	10,954.90				

but not for the interaction ($p = 1.000$). The predicted model can thus be applied. Because no residual error was found (see Table 3c), means that the model can explain the variation in the response.

Conclusions

Results indicate that ionic strength does not modify the interaction between haze precursors and tannic acid. Proteins contribute substantially to haze formation, although contribution of polysaccharides is also significant. Polysaccharides combine with proteins but not with polyphenols. The response surface methodology (RSM) was useful (i) to explain the influence of both variables and (ii) to suggest a more appropriate adjustment model. However, it is possible that polysaccharides do not require an increased use of stabilizing agents or they have no influence. Therefore, using tannic acid as an indicator of the interaction between polysaccharides and proteins, as commonly done, could lead to considerable errors. In fact, not only the protein but also the polysaccharides react with tannic acid and, in this way, actually cause a considerable increase in turbidity that should be taken into account.

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References

Atkinson, S. (2005). Beer clarification. *Membrane Technology*, *2005*, 8–9.
Bamforth, C. (2009). *Beer: A quality perspective* (1st ed.). New York: Academic Press, Inc
Benitez, E. I., Genovese, D. B., & Lozano, J. E. (2007). Effect of pH and ionic strength on apple juice turbidity: Application of the extended DLVO theory. *Food Hydrocolloids*, *21*, 100–109.
Benitez, E. I., Genovese, D. B., & Lozano, J. E. (2007). Scattering efficiency of a cloudy apple juice: Effect of particles characteristics and serum composition. *Food Research International*, *40*, 915–922.
Benítez, E. I., & Lozano, J. E. (2007). Effect of gelatin on apple juice turbidity. *Latin American Applied Research*, *37*, 261–266.
Benitez, E. I., Lozano, J. E., & Genovese, D. B. (2010). Fractal dimension and mechanism of aggregation of apple juice particles. *Food Science and Technology International*, *16*, 179–186.
Benítez, E., Martinez Amezaga, N., Sosa, G., Peruchena, N., & Lozano, J. (2013). Turbidimetric behavior of colloidal particles in beer before filtration process. *Food and Bioprocess Technology: An International Journal*, *6*, 1082–1090.
Bradford, M. (1976). A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein–dye binding. *Analytical Biochemistry*, *72*, 248–554.
Briggs, D. E., Boulton, C. A., Brookes, P. A., & Stevens, R. (2004). *Brewing science and practice.* Cambridge, UK: Woodhead Publishing Limited.
Buiatti, S. (2009). Beer composition: An overview. In V. R. Preedy (Ed.), *Beer in health and disease prevention* (pp. 212–225). London: Academic Press.
Dickinson, E. (1994). Colloidal aspects of beverages. *Food Chemistry*, *51*, 343–347.
Dobbins, R., & Jizmagian, G. (1966). Optical scattering cross sections for polydispersions of dielectric spheres. *Journal of the Optical Society of America*, *56*, 1345–1350.
Fleet, C., & Siebert, K. (2005). Effect of illumination intensity on visual perception of turbidity. *Food Quality and Preference*, *16*, 536–544.
Kumar, A., Prasad, B., & Mishra, I. M. (2008). Optimization of process parameter for acrylonitrile removal by low cost adsorbent using Box–Behnken design. *Journal of Hazardous Materials*, *150*, 174–182.
McClements, D. (2005). *Food emulsions: Principles, practice, and techniques.* London: CRC Press.
McMurrough, I., Madigan, D., Kelly, R. J., & O'Rourke, T. (1999). Haze formation and shelf-life prediction for lager beer. *Food Technology*, *53*(1), 58–62.
MINITAB® Release 15 Statistical Software for Windows, Minitab Inc., USA (2006).
Mitchell, A., Hong, Y. -J., May, J. C., Wright, C., & Bamforth, C. (2005). A comparison of polyvinylpolypyrrolidone (PVPP), silica xerogel and a polyvinylpyrrolidone (PVP)–silica co-product for their ability to remove polyphenols from beer. *Journal of the Institute of Brewing*, *111*, 20–25.
Montgomery, D. C. (2003). *Design and analysis of experiments.* New York: Wiley and Sons.
Oh, H. I., Hoff, J. E., Armstrong, G. S., & Haff, L. A. (1980). Hydrophobic interaction in tannin–protein complexes. *Journal of Agricultural and Food Chemistry*, *28*, 394–398.
Segarra, I., Lao, E., López-Tamames, E., & De la Torre-Boronat, M. C. (1995). Spectrophotometric methods for the analysis of polysaccharides levels in winemaking products. *American Journal of Enology and Viticulture*, *46*, 564–570.
Siebert, K. J. (1999). Effects of protein–polyphenol interactions on beverage haze, stabilization and analysis. *Journal of Agricultural and Food Chemistry*, *47*, 353–362.
Siebert, K. (2006). Haze formation in beverages. *Food Science and Technology*, *39*, 987–994.
Siebert, K. (2009). Haze formation in beverages. *Advances in Food and Nutrition Research*, *57*, 53–86.
Siebert, K., Carrasco, A., & Lynn, P. (1996). Formation of protein–polyphenol haze in beverages. *Journal of Agricultural and Food Chemistry*, *44*, 1997–2005.
Singleton, V. L., Orthofer, R., & Lamuela-Raventos, R. M. (1999). Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin Ciocalteu reagent. *Methods in Enzymology*, *299*, 152–178.
Steiner, E., Becker, T., & Gastl, M. (2010). Turbidity and haze formation — Insights and overview. *Journal of the Institute of Brewing*, *116*, 360–368.
Thomas, N., Coakley, W., & Winters, C. (1996). Contact formation in polylysine-mediated membrane–glass interaction. *Colloids and Surfaces B: Biointerfaces*, *6*, 139–147.
Van Buren, J. P. (1989). Causes and prevention of turbidity in apple juice. In D. L. Downing (Ed.), *Processed apple products.* New York: Van Nostrand Reinhold.
Van der Sman, R. G. M., Vollebregta, H. M., Mepschen, A., & Noordman, T. R. (2012). Review of hypotheses for fouling during beer clarification using membranes. *Journal of Membrane Science*, *396*, 22–31.