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Analytical Methods

Characterisation of sparkling cider by the yeast type used in taking foam on the basis of polypeptide content and foam characteristics

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ABSTRACT

This paper describes the characterisation of sparkling ciders from Asturias, northern Spain, by means of the analysis of their protein content and their foam characteristics. A capillary sieving electrophoretic method was used in the protein analysis to determine molecular mass and the Bradford method to determine total protein content. The foam parameters were measured using the Bikerman method.

Chemometric techniques, such as principal component analysis (PCA) and soft independent modelling of class analogy analysis (SIMCA), allowed the sparkling cider to be differentiated on the basis of the yeast strain used in their manufacture. As a result, feasible models for classifying sparkling ciders were computed (classification hits higher than 96%).

Furthermore, the relationship between polypeptides and foam was demonstrated with a prediction equation of foam stability time, which was computed using the partial least square (PLS) regression technique. This mathematical equation confirmed that the polypeptides of high molecular mass are especially related to this foaming parameter.

1. Introduction

In April 2005, the cider produced in Asturias achieved the Protected Designation of Origin "Sidra de Asturias" (Council Regulation (EEC) No. 2081/92, 2005); this quality mark includes two kinds of beverages made exclusively from Asturian apples and with endogenous carbonic gas: "Natural Cider" and "Cider". The former is the more popular in Asturias and includes one open fermentation step without any further clarification or filtration procedure. "Cider" is a sparkling beverage which is filtered and which presents higher levels of CO_2 than "Natural Cider". Several methods are employed to make this sparkling cider, one of the most popular being the second fermentation in bottle technology.

Foam is one the most important characteristics of cider due to being the first attribute that consumers perceive. The typical visual attributes assessed in sparkling ciders are: initial foam, foam area persistence, number of nucleation sites, bubble size and foam collar (Picinelli Lobo, Fernández Tascón, Rodríguez Madrera, & Suárez Valles, 2005).

Several studies have concluded that proteins, polysaccharides and fatty acids are important molecules in the constitution and stabilization of foam. In sparkling wines, foam stability is positively related to protein concentration (Pueyo, Martín-Álvarez, & Polo, 1995). The presence of polysaccharide–polypeptide complexes at the protein isoelectric point improves foam stability, since interactions increase and electrostatic repulsions decrease, thus facilitating the formation of a viscoelastic film that reduces drainage of the liquid film. Moreover, the effect of foam-negative materials such as fatty acids may be inhibited by various proteins isolated from beer which can bind lipids and improve foam stabilization (Cooper, Husband, Mills, & Wilde, 2002). On the other hand, Mangas, Moreno, Rodríguez, Picinelli, and Suárez (1999) reported the effect of methanol (a molecule linked to pectic substances in apples) and acidic polysaccharides on cider foam stability, which could be related to an increase in viscosity that reduces drainage of the liquid film (Prins, 1988), and also indirectly reduces the degree of disproportionation of bubbles (Ostwald ripening) (Bamforth, 2004). Likewise, Margolles-Cabrales (2002) established significant mathematical correlations between foam characteristics and the fatty acids composition of cider.

The chemical and physical characteristics of proteins influence the formation of foam and its stabilization. Thus, for instance, proteins with good foamability tend to be flexible and relatively small, on the contrary, proteins with good foam stability are able to cross-link and aggregate and are resistant to mechanical deformation (Bamforth, 2004). In addition, Brissonnet and Maujean (1993) established that hydrophobic proteins contribute more to foam constitution than hydrophilic ones.

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To carry out a study on the foam of a sparkling beverage, it is necessary to measure its foaming properties: foam height (FH), foam stability height (FS) and foam stability time (ST). Foamability is determined by the FH parameter, FS characterises the average lifetime of the bubbles, which is related to collar quality, and ST represents the average lifetime of the foam after gas injection has ceased.

Picinelli Lobo et al. (2005) demonstrated the influence of ageing time and yeast strain on the foaming properties of sparkling ciders. The yeast strain significantly influenced foamability and Bikerman's coefficient (Bikerman, 1938), while ageing time significantly influenced foamability and foam stability time. The influence of grape variety, base wine and ageing time on the foaming properties of sparkling wines was likewise studied by Andrés-Lacueva, Gallart, López-Tamames, and Lamuela-Raventós (1996), Andrés-Lacueva, López-Tamames, Lamuela-Raventós, Buxaderas, and Torre-Boronat (1996) and Andrés-Lacueva, Lamuela-Raventós, Buxaderas, and Torre-Boronat (1997). These authors concluded that ageing time improved the stability time of the foam collar and that the best foamability and stability time is achieved at 18 months of ageing, probably due to release of proteins and polysaccharides by yeast autolysis.

This paper reports the study of the proteins and the foam characteristics (FH, FS, and ST) of the sparkling cider fermented by the method of second fermentation in bottle. With this purpose in mind, a capillary electrophoretic system developed by our research group (Blanco, Junco, Expósito, & Gutiérrez, 2003, 2004) was used to separate the sodium dodecyl sulphate (SDS)–protein complexes in a sieving matrix of linear polyacrylamide. The goal of this study was to determine the molecular masses of the protein profiles and the foam properties of sparkling ciders in order to carry out their characterisation. Chemometric techniques had to be applied to extract the information contained in the high number of observations obtained from the analytical processes. In fact, with such number of analytical data, the multivariate techniques are the most appropriate statistical methodologies for classifying purposes. For example, the principal component analysis (PCA) analysis was used to detect some kind of grouping within the ciders studied and the soft independent modelling of class analogy analysis (SIMCA) was used to classify the samples. Finally, the partial least square (PLS) regression technique was used to establish mathematical equations that describe the relations amongst the variables studied.

This work completes the first paper about proteins and foam properties in cider carried out by our research group (Blanco-Gomis, Mangas-Alonso, Junco-Corujedo, & Gutiérrez-Álvarez, 2007). In that first work the variation sources studied were the sensory evaluation and the press technology, and the cider analysed was "Natural Cider", the other one included in the Protected Designation of Origin "Sidra de Asturias", which is a still beverage with different foam characteristic from the "Cider".

2. Materials and methods

2.1. Samples

Sparkling ciders ($n = 29$) were made at the "Sidra el Gaitero" cellar in two vintages (2001–2002 and 2002–2003). The procedure of making the sparkling cider was as follows. Asturian cider apples were washed and milled. The pulp was then macerated for 9–12 h and subsequently pressed in a hydraulic press. Fermentation was conducted by wild microflora in stainless steel casks at 14 °C–20 °C. When the first fermentation ended, the cider was matured until obtaining optimal sensory properties of the cider (base cider). The base cider was then clarified through a 0.22 μm pore size ceramic microfilter. In order to carry out the foam taking in bottle, 18 g/L sucrose; 0.05%(w/v) nutritive solution (ammonium sulphate 96% (w/w), citric acid 3.3%(w/w) and thiamine 0.7% (w/w)); and 0.003% (w/v) bentonite, used as fining agent, were added at the base cider. This cider was inoculated (2%) with two yeast types: cider yeast (C6, *Saccharomyces bayanus*) belonging to the SERIDA yeast collection or a commercial winery yeast (Levuline CHP, *Saccharomyces cerevisiae*). Finally, the cider was bottled (bottles of 0.75 and 1.5 L), and the second fermentation and ageing "surlie" was conducted at 12 °C–15 °C over a period of 17 months. Sampling commenced once the foam taking was done and continued during the ageing time in bottle for 16 months.

2.2. Electrophoretic analysis

Electrophoretic analyses were carried out on a HewlettPackard (Waldbronn, Germany) ^{3D}CE apparatus equipped with a diode array detector and a HP ChemStation software package. Fused silica capillary tubes with 100 μm internal diameter were purchased from Tecknokroma (Barcelona, Spain). These capillaries were filled with linear polyacrylamide and a background electrolyte of Tris plus aspartic acid.

Reversed polarity at 20 kV and hydrodynamic injection (50 mbar, 50 s) were used in the analysis of the SDS–protein complexes. Detection was performed at 220 nm.

The samples were treated as follows. The cider was sonicated, centrifuged and filtered before being purified and concentrated (50 times) in the Ultrafree® Centrifugal filter device of 5 kDa Molecular Weight Cut-Off, purchased from Millipore (Billerica, USA). A solution of SDS and Tris was added to the concentrate, which was then boiled with 2-mercaptoethanol for 10 min (Blanco et al., 2003, 2004). The molecular weight standard proteins (aprotinin, 7 kDa; lysozyme, 14.5 kDa; trypsin inhibitor, 21 kDa; carbonic anhydrase, 31 kDa; ovalbumin, 45 kDa; bovine albumin or BSA, 66.5 kDa; and β-galactosidase, 112 kDa) correspond with the range of molecular weight found in cider; they were dissolved in the SDS–Tris solution, and boiled as the sample.

2.3. Measurement of foam properties

Bikerman's method (Bikerman, 1938) was adapted to analyse foaming parameters in cider. A 50 mL (60 × 1 cm) glass column fitted at the bottom with a sintered glass disc (pore size 10 μm) was used. The gas flow (carbon dioxide) was controlled by means of a pressure regulator. Cider (10 mL), previously degasified (2 min under vacuum and shaking) at 20 °C, were poured into the column. Carbon dioxide was sparged through the cider at a flow rate of 30 mL/min. Maximum height (FH parameter) and stable height (FS parameter) were recorded while maintaining a continuous flow of carbon dioxide. The CO_2 flow was then interrupted and the time elapsed (ST parameter) until all the bubbles collapsed was measured. The column was washed before each experiment with 10 mL ethanol 95%, three times with 10 mL Milli-Q water and finally with 25 mL of cider sample. Analyses were carried out in triplicate.

2.4. Total protein analysis

Quantitative determination of the total protein content was carried out by means of the "Bradford" assay (Bradford, 1976). This assay is based on the specific binding of the Coomassie Brilliant Blue-G250 dye to protein molecules. This organic dye binds to tyrosine side chains, giving a dye–protein complex with an absorption maximum of 595 nm.

Cider samples were purified before analysis in the Ultrafree® Centrifugal filter device, subsequently dissolving the concentrate in water until obtaining the initial volume. The concentrate

Bradford dye reagent of Bio-Rad (USA) (Bradford microassay) was added to the samples and the standard in the same amount. The calibration curve was built using bovine serum albumin as the standard and a detection wavelength of 595 nm was set.

2.5. Database and statistics

The database consisted on 29 rows (sparkling ciders) and 22 columns (polypeptides). Ciders were categorised in two classes on the basis of the yeast type used in the foam taking: cider yeast (*S. bayanus*) *vs*. winery yeast (*S. cerevisiae*). Fifteen ciders were made using the *S. bayanus* strain (cider category, C), and 14 were made using the *S. cerevisiae* strain (wine category, W).

Multivariate analyses (principal component analysis, soft independent modelling of class analogy, correlation analysis, and partial least squares regression) were carried out using the PARVUS statistical package (Forina, Leardi, Armanino, & Lanteri, 1988). Data were autoscaled before multivariate analysis.

3. Results and discussion

3.1. Univariate analysis of the total protein content

The total protein content was analysed by the Bradford method in ciders previously purified by ultracentrifugation with 5 kDa molecular weight cut-off membranes; thus only polypeptides with molecular masses higher than 5 kDa were quantified. The mean value for the ciders fermented with the winery yeast was 8.9 mg/L and for the other group of ciders the mean was 10.0 mg/L. The ANOVA was carried out with these data and no statistically significant difference exists between the means of total protein at the 95% confidence level. However, should be noted that there is a clear difference with respect to the total protein concentration of "Natural Cider", in which our research group detected levels of protein of around 20 mg/L (Blanco-Gomis et al., 2007). The reason for the decrease in protein from one kind of cider to another could be the consumption of this substance by the yeast added for the second fermentation. However, the effect of bentonite in the decrease of the protein content is probably much higher than the proteolytic activity of the yeast. This is due to the electrostatic interaction of bentonite with proteins and some authors have described this effect in sparkling wines (Martinez-Rodríguez & Polo, 2003; Vanrell et al., 2007).

To sum up, we can state that the two kinds of yeast produce a similar effect on the total protein content of sparkling cider.

3.2. Factor analysis of the internal structure of the polypeptide database

As can be appreciated in Fig. 1, electrophoretic analysis detected 22 peaks that were identified as polypeptides, with molecular masses ranging between 7.2 and 102.1 kDa. The result is that there are 10 polypeptides less in the sparkling cider than in the Natural Cider and the maximum molecular mass is lower, when compared to the 154 kDa polypeptide found in the latter cider (Blanco-Gomis et al., 2007). This effect seems to be related to the decrease in protein content caused by the addition of bentonite and yeast (less important) in the second fermentation.

Multivariate analyses were carried out on the 22 polypeptides to detect some kind of structure or differentiation between the two types of yeast used to make the cider. Before this analysis, however, we decided to reduce the number of variables, removing a number of highly correlated polypeptides which can add noise to the constructed models and deteriorate the classification of the samples. The correlations between original variables (polypeptides) were computed at a confidence level of 90%, the criterion employed being to remove one variable from each pair of polypeptides with correlation coefficients higher than 0.81 in absolute values. Accordingly, the following variables were removed: P2 (11.0 kDa), P7 (16.9 kDa), P15 (49.4 kDa), P19 (84.8 kDa), and P20 (90.3 kDa), resulting in a new database matrix of 29 rows (sparkling ciders) $\times$ 17 columns (polypeptides).

Principal component analysis (PCA) was carried out to reduce the dimensionality of the database and to ascertain its structure. Five significant principal components, which accounted for 81.32% of the variance (eigenvalues higher than 1.0), were chosen. Fig. 2 shows a projection of the sparkling ciders and the polypeptides on the plane formed by the second and fifth principal components (total accumulated variance, 26.96%). As can be seen in Fig. 2, a data structure is visualised. Most of the sparkling ciders made with the winery strain (W in the figure) are placed in the upper right corner of the factorial plane, detecting relations with two polypeptides of medium molecular mass (P12, 35.1 kDa, and P17, 69.2 kDa), and two polypeptides of low molecular mass (P1, 7.2 kDa; and P4, 13.5 kDa). However, the sparkling ciders made with the cider strain (C in the figure) are mainly placed in the upper left corner of the factorial plane, being related to most of the polypeptides, especially to those of high and medium molecular masses (for example, P22, 102.1 kDa; P18, 73.0 kDa; P16, 51.7 kDa, P14, 43.9 kDa; P11, 22.8 kDa; P10, 21.4 kDa and P8, 19.8 kDa). In addition, there are some polypeptides of low molecular mass that are highly related to these ciders, such as P3 (12.1 kDa) or P5 (14.3 kDa). The relationship detected in the PCA analysis between sparkling ciders made with the winery yeast strain and polypeptides with low molecular size could justify the higher significant foamability demonstrated by Picinelli Lobo et al. (2005) in these ciders, compared to those made with the cider yeast strain, since proteins with good foamability and probably with bad foam stability tend to be relatively small (Bamforth, 2004).

Therefore, PCA analysis showed that the yeast used in the second fermentation has an effect on the electrophoretic profile, the cider yeast being the one that produced higher concentrations of polypeptides of high and medium molecular mass, just the contrary to the other yeast.

3.3. Soft independent modelling of class analogy (SIMCA)

Once the data structure had been visualised, we were able to carry out modelling analysis to compute feasible mathematical models that allowed us to classify sparkling ciders on the basis of the yeast strain used in the foam taking process and ageing "surlie" time. With this purpose in mind, we computed a SIMCA reduced model with four principal components that accounted for 84% and 83% of the variance for the models W (winery strain) and C (cider strain), respectively. Fig. 3 shows a Coomans' diagram displaying the two SIMCA boxes. As can be seen, classification hits are 96.6%, since only one sparkling cider (* in Fig. 3) belonging to class C is classified as belonging to class W. Sensitivities for the models were 92.9% (W model) and 93.3% (C model). Thus, approximately only 7% of samples (‡ in Fig. 3) belonging to each model are rejected from their own model. The specificity of model C was 100%, since no sample belonging to model W is accepted in model C. The specificity of model W was lower (86.7%); 13% of samples belonging to model C († in Fig. 3) are accepted in model W. The most discriminating variables were P17 (69.2 kDa) and P3 (12.1 kDa). These results are in agreement with PCA analysis (see Fig. 2), since polypeptide P3 presents the highest loading (−0.45) in absolute value for the second principal component, and polypeptide P17 presents one of the highest loading values (0.35) for the fifth principal component. P17 is one of the polypeptides asso-

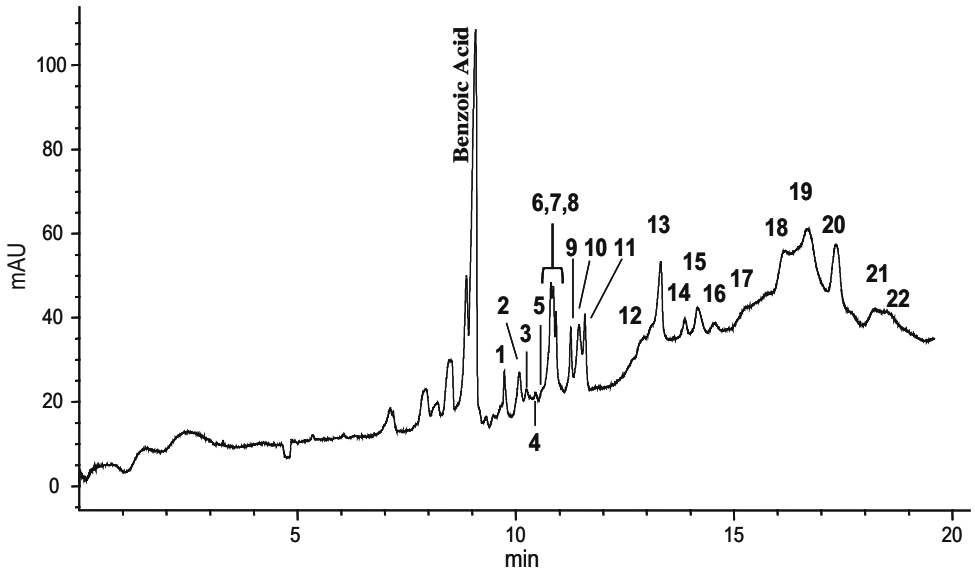

Fig. 1. Eletrophoretic polypeptide profile of a sparkling cider. Each polypeptide is numbered consecutively in order of molecular weight.

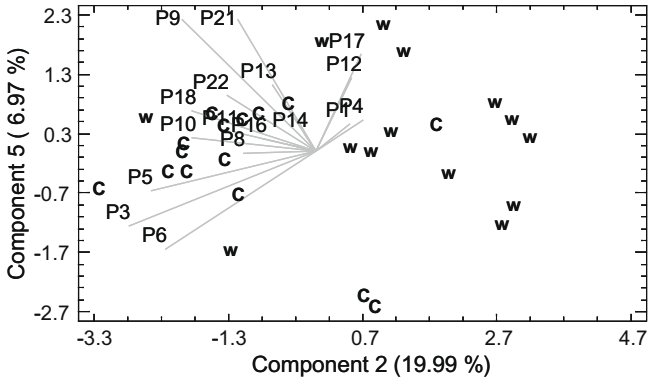

Fig. 2. Projection of the sparkling ciders and polypeptides (P_i) onto the plane formed by the second and fifth predictive principal components. W: sparkling ciders made with the winery strain; C: sparkling ciders made with the cider strain.

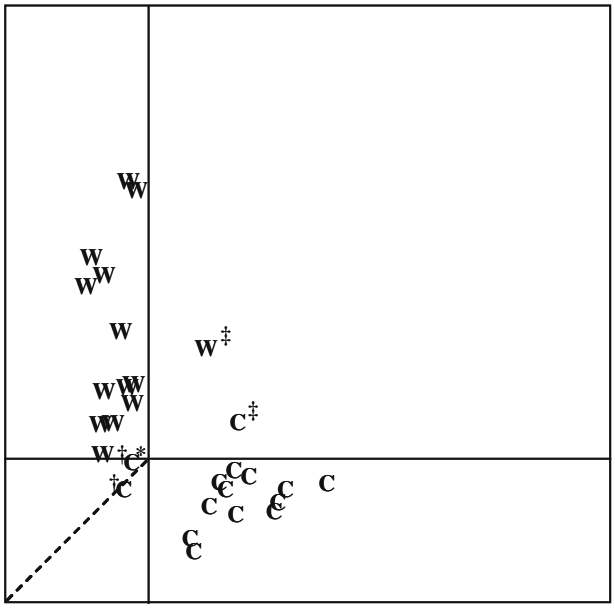

Fig. 3. Cooman's diagram for SIMCA analysis. W: wine class; C: cider class.

ciated with sparkling ciders made with the winery yeast strain (see Fig. 2), and P3 with sparkling ciders made with the cider yeast strain (see Fig. 2).

In conclusion, SIMCA analysis in combination with electrophoretic analysis of polypeptides based on their molecular size is able to differentiate sparkling ciders by the yeast strain used in the foam taking.

3.4. Pearson correlation analysis between polypeptides and foam parameters

Foam stability time (ST) is the parameter most influenced by polypeptides, since there are more statistically significant correlations coefficients between them and this foam parameter than between the other two. Furthermore, those polypeptides with low molecular size (less than 39 kDa) show negative correlation coefficients (−0.470; $p < 0.01$; for polypeptide P10 of 21.4 kDa) with foam stability time, precisely the opposite effect being observed for polypeptides with higher molecular mass (0.718; $p < 0.001$; for polypeptide P17 of 69.2 kDa). The parameter related to the average lifetime of bubbles (FS) was positively correlated with many polypeptides ranging from 19.8 to 96.0 kDa, with coefficients around 0.55. Finally, the parameter that represents foamability (FH) was less related to the polypeptides found in the ciders than the other two foam parameters, since no significant correlation was found.

Summarizing, we can state that polypeptides of high molecular mass help to stabilize foam; those of low molecular mass influence negatively in the foam stabilization; those of medium and high molecular mass increase bubble lifetime; and none of them seems to have much relation to foamability.

3.5. Partial least squares (PLS) regression

PLS regression allows us to predict a set of dependent variables (criterion or response variables) from a (very) large set of independent variables (predictor variables), and to describe their common structure. In our case, the set of dependent variables were the foaming properties (FH, FS, and ST), while the independent variables were the polypeptides ($p = 22$). The database consisted of a matrix of 29 (sparkling ciders) × 25 (predictor and response

Table 1
PLS parameters for foaming properties: foam height (FH), foam stability height (FS), and foam stability time (ST).

Foaming property	Latent variables number	Cross-validated explained variance (%)	Explained variance (%)	Multiple linear correlation coefficient (%)
FH	5	21.4	45.3	55.1
FS	5	26.6	53.0	61.4
ST	2	61.4	69.6	71.7

Table 2
Importance value for each polypeptide selected by means of the PLS model, and coefficients of each of these in the prediction equation of foam stability time.

Polypeptide	Equation coefficient	Importance
P1	−123.2	0.0240
P3	−162.4	0.1085
P4	30.5	0.0051
P6	−223.7	0.0753
P13	−24.0	0.0271
P14	45.3	0.0544
P15	71.4	0.0599
P16	339.5	0.1570
P17	130.8	0.1989
P18	−59.8	0.0886
P20	20.3	0.0750
P21	163.6	0.1263

variables). Predictor variables were chosen by their weighted importance parameter, the cut-off value chosen for this parameter being 0.03. With this criterion in mind, the following polypeptides were selected (kDa): P1 (7.2); P3 (12.1); P4 (13.5); P6 (15.8); P13 (38.3); P14 (43.9); P15 (49.4); P16 (51.7); P17 (69.2); P18 (73.0); P20 (90.3); and P21 (96.0). Table 1 shows the PLS parameters for each foaming property. The number of latent variables was optimised, maximising cross-validated explained variance. foam stability time showed the highest cross-validated explained variance and the highest multiple linear correlation coefficient. As can be seen in Table 2, the most important predictor variables in order of importance in the PLS model were the following: P17 > P16 > P21 > P3. This table also shows the equation coefficients of the prediction equation for foam stability time. As we can see, P21, P16 and P17 have positive coefficients, while P3 has a negative coefficient. To complete the analysis, the regression equation that relates the foam stability time values predicted by the PLS model (ST_{pre}) with the experimental values (ST_{exp}) of this foaming property was calculated ($ST_{exp} = ST_{pre} * 0.82 + 4.34$), resulting in a $r^2 = 0.82$; which implies a high level of correspondence between the two sets of values. Therefore the computed PLS model confirms that polypeptides with high molecular mass positively influence foam stability time, which is, at the same time, the foam parameter most affected by polypeptides. These results are also in agreement with the hypothesis that polypeptides of small molecular size destabilize foams but contribute to improve foamability, although in our case we did not find good correlations between the FH parameter and polypeptides.

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