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Synergistic Effect of High and Low Molecular Weight Molecules in the Foamability and Foam Stability of Sparkling Wines

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ABSTRACT: The foam of sparkling wines is a key parameter of their quality. However, the compounds that are directly involved in foam formation and stabilization are not yet completely established. In this work, seven sparkling wines were produced in Bairrada appellation (Portugal) under different conditions and their foaming properties evaluated using a Mosalux-based device. Fractionation of the sparkling wines into four independent fractions, (1) high molecular weight material, with molecular weight higher than 12 kDa (HMW), (2) hydrophilic material with molecular weigh between 1 and 12 kDa (AqIMW), (3) hydrophobic material with molecular weigh between 1 and 12 kDa (MeIMW), and (4) hydrophobic material with a molecular weight lower than 1 kDa (MeLMW), allowed the observation that the wines presenting the lower foam stability were those that presented lower amounts of the MeLMW fraction. The fraction that presented the best foam stability was HMW. When HMW is combined with MeLMW fraction, the foam stability largely increased. This increase was even larger, approaching the foam stability of the sparkling wine, when HMW was combined with the less hydrophobic subfraction of MeLMW (fraction 3). Electrospray tandem mass spectrometry (ESI-MS/MS) of fraction 3 allowed the assignment of polyethylene glycol oligomers (n = 5−11) and diethylene glycol 8-hydroxytridecanoate glyceryl acetate. To observe if these molecules occur in sparkling wine foam, the MeLMW was recovered directly from the sparkling wine foam and was also analyzed by ESI-MS/MS. The presence of monoacylglycerols of palmitic and stearic acids, as well as four glycerylethylene glycol fatty acid derivatives, was observed. These surface active compounds are preferentially partitioned by the sparkling wine foam rather than the liquid phase, allowing the inference of their role as key components in the promotion and stabilization of sparkling wine foam.

KEYWORDS: foam, sparkling wines, mass spectrometry, glycerol derivatives, ethylene glycol, tensioactives, surfactants

INTRODUCTION

According to the traditional method, sparkling wine is a double-fermented wine. In this method, the wine obtained by the fermentation of the must (base wine) is submitted to a second alcoholic fermentation by addition, in the bottle, of a suspension of yeast and sugar. When poured from the bottle into a glass, the carbon dioxide produced during the second fermentation is released from the liquid in the form of bubbles and by diffusion through the free air/liquid interface.[1] Consistent foam is formed as a result of its interaction with wine constituents.

Foam is the dispersion of a gas in a continuous liquid phase, and thus foam dispersions possess bulk densities closer to those of a gas rather than a liquid. Foam is stable if gas bubbles remain separated by thin liquid walls and do not coalesce. Drainage, the runoff of liquid between bubbles in foam, is dependent not only on the liquid viscosity and density but also on the presence of a layer of surface active molecules adsorbed on the air/liquid interfaces of both films, which separate the bubble from ambient air.[2] The chemical composition of induced base wine foam is less acidic than the bulk liquid phase due to the lower concentration of organic acids and higher in protein and polysaccharides,[3] as well as free fatty acids (C6:0−C16:0) and their ethyl esters (C8:0 and C10:0).[4]

The foam properties, foamability and foam stability, have been correlated with the sparkling wine chemical composition, namely, soluble proteins,[5,6] polysaccharides,[7,8] polyphenols,[5,8] iron,[3] organic acids, and lipids.[9] Proteins were the first candidates to be correlated with foam characteristics due to their surfactant properties. Surfactant agents are inferred to stabilize foams by settling at the bubble's edge, with the hydrophobic side interacting with the gas phase and the hydrophilic side interacting with the aqueous liquid phase.[3] Protein concentration was previously positively correlated with foamability by several authors;[3,6,10,11] however, correlation with foam stability has presented contradictory results, with both positive[6] and negative correlations.[12] All of these data were obtained by measuring the foamability and foam stability of base[3−12] or sparkling wines[7,10] and relating these physical characteristics with the chemical characteristics of sparkling wines with different foam properties.

Peptides have also been associated with the foam characteristics, namely, the amphiphilic low molecular weight peptides.[13] The presence of aromatic amino acids (that confer hydrophobicity to the peptides) in Cava sparkling wines has also been shown to improve the quality of the foam of these wines.[14] Contrarily, Moreno-Arribas et al.[7] did not find any relationship between foam characteristics and concentrations of wine peptides.

In addition to peptides and proteins, some polysaccharide fractions were also correlated with foamability, and a fraction with 2−3 kDa was correlated with foam stability.[15] Neutral polysaccharides were well correlated with foamability in opposition to acidic polysaccharides, which did not show any correlation with foamability.[7] Lipids have also been correlated with foam properties: palmitic acid was positively correlated with foamability,[16] as well as fatty acids esterified with ethanol; however, a

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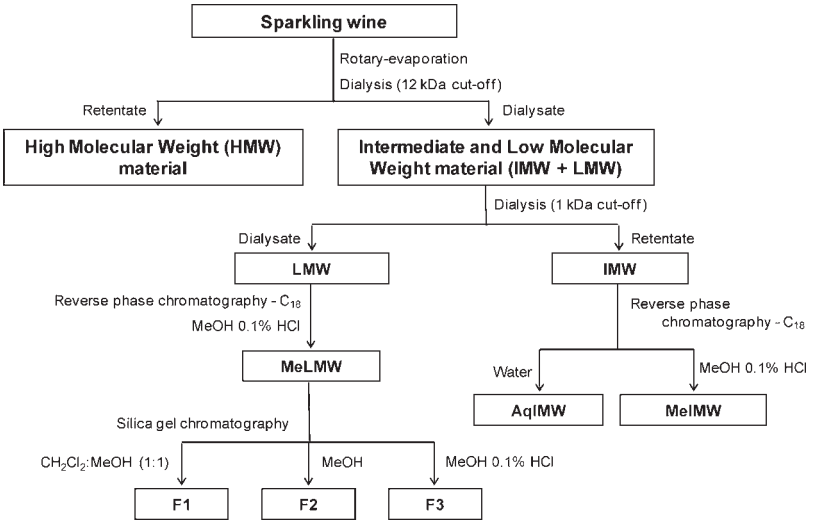
Scheme 1. Schematic Diagram of the Isolation and Fractionation Steps of Sparkling Wines



negative correlation with foamability was obtained for fatty acids under C_{12} in free form.[4]

Mass spectrometry (MS) techniques, and particularly soft ionization methods, allow the analysis of low molecular weight compounds from food matrices. Soft ionization mass spectrometry has been used for the analysis of a large number of low molecular weight compounds, including peptides,[17] oligosaccharides,[18] lipids,[19] and ionic and nonionic surfactants.[20,21] Ultra-high-resolution MS (FT-ICR-MS) was used to discriminate surface active components from Champagne aerosols and bulk.[22]

To study the synergistic effect of high and low molecular weight molecules in the foamability and foam stability of sparkling wines, in this work, the foam aptitude of seven sparkling wines was evaluated. These sparkling wines were then fractionated into four independent fractions according to their molecular weight and hydrophobicity, and the amount of material in these fractions was related with their foaming properties. The fractions obtained with the highest foam stability were then used to reconstitute wine model solutions to evaluate the individual contribution of each fraction to foam properties. The fraction showing the highest influence on the foam stability was structurally characterized by electrospray tandem mass spectrometry ($ESI\text{-}MS^n$). To observe if the molecules identified as major contributors to sparkling wine foam stabilization are really present in sparkling wine foam, the foam was collected and its low molecular weight material was also structurally characterized by ESI-MS and ESI-MS/MS.

MATERIALS AND METHODS

Sparkling Wine Samples. Sparkling wines were prepared by Estação Vitivinícola da Bairrada (EVB) from two grape varieties, that is, Fernão-Pires (FP) white variety and Baga (BG) red variety, obtained from different ripening stages and soils. To produce FP wines, grapes from a clayey (C) soil were picked at three harvest moments: (1) at adequate harvest maturity (A) to produce sparkling wines (FP_{AC}), determined by berry texture, color, sugar content, and titratable acidity; (2) at an early harvest moment (E), 1 week before maturity harvest (FP_{EC}); and (3) at a late harvest moment (L), 1 week after maturity harvest (FP_{LC}). FP wines were also produced from grapes collected in soils presenting different textures: sandy (S) (FP_{AS}) and clay−calcareous (CC) soils (FP_{ACC}). BG sparkling wine was produced from ripe grapes (adequate harvest moment to produce sparkling wines determined by the physicochemical parameters) and one soil type, clay (BG_{AC}). A mixture of musts (50:50) obtained from BG and FP grapes picked at the harvest moment from clayey soil was also used to produce sparkling wines ($FP_{AC}+BG_{AC}$). The sparkling wines were produced according to the traditional method, and two independent winemaking replicates were performed for each type of wine (FP_{EC}, FP_{AC}, FP_{LC}, FP_{AS}, FP_{ACC}, and BG_{AC}). The second fermentation was performed inside the bottles after *tirage*, and at least four different bottles were analyzed for each type of wine, in a total of 24 bottles. The exception of this strategy was the mixture $FP_{AC}+BG_{AC}$, for which only two bottles were obtained. The wines were aged for 12 months on yeast lees, and the *dégorgement* (removal of yeast sediment from bottles) occurred after that period of time. The wines were analyzed 24 months after *dégorgement*. Each bottle was analyzed in duplicate.

Extraction of Polymeric Material from Sparkling Wines. The sparkling wine samples were rotary-evaporated under reduced pressure at 35 °C to degas and eliminate the ethanol, allowing the nonvolatile molecules to be concentrated. The material was then dialyzed (12 kDa cutoff membrane, Medicell) to remove the tartaric acid and other small molecules. The retentate was concentrated, frozen, and freeze-dried, to give the wine high molecular weight (HMW) material as a powder (Scheme 1). The material that diffused through the dialysis membrane (dialyzate) was recovered by concentration under rotary evaporation and frozen for use in the following isolation step.

Extraction and Isolation of Intermediate and Low Molecular Weight Material from Sparkling Wines. The different concentrated 12 kDa dialyzed solutions obtained during the isolation of the polymeric material of each sparkling wine were then submitted to a new dialysis, now with a cutoff of 1 kDa (Spectra/Por) (Scheme 1). Each aqueous solution was added, under stirring, to a batch of a C_{18} resin suspension, during 3 h, for adsorption of the hydrophobic material. The resin was recovered by filtration, washed with water until the conductivity of the water is reached, and then extracted with acidic methanol (MeOH 0.1% v/v HCl). Using this procedure, the retentate, which comprised the material with molecular weight between 1 and 12 kDa (IMW), gave rise to two fractions, AqIMW, the fraction of material not sorbed to the C_{18}, which remained in the water solution, and MeIMW, the fraction of material retained in the C_{18} resin and recovered with acidic methanol (Scheme 1). The dialysate, which comprised the material with molecular weight lower than 1 kDa, gave rise to fraction

MeLMW, extracted with methanol; the fraction not sorbed, containing the salts, was discarded.

The fraction MeLMW was then fractionated by polarity through a silica column using the following sequence of eluents: CH_2Cl_2/MeOH (1:1), MeOH, and acidic MeOH (0.1% HCl, v/v), giving rise to fractions F1, F2, and F3, respectively (Scheme 1).

Extraction of Foam Low Molecular Weight Material. The fraction MeLMW was also extracted from sparkling wine foam of FP_{LC} sample. The foam formed by uncorking the bottle (750 mL) and then by bottle agitation was collected (100 mL of collapsed foam) and dialyzed (1 kDa cutoff membrane, Spectra/Por) against water (1 L) at 5 °C, under stirring, until the conductivity of the dialysis water became similar to that of distilled water (one water exchange of 1 L each, during 48 h). The two dialysates (containing the lower molecular weight material) were combined and eluted by a C_{18} column (SPE-C_{18}, Supelco-Discovery 10 g). Then, the retained material was washed with ultrapure water, until the water conductivity reached 2.3 μS/cm, and the foam low molecular weight hydrophobic material was recovered with acidic methanol (1.0% v/v acetic acid). The sample was concentrated by rotary evaporation at 35 °C and suspended in ultrapure water. The solution was centrifuged, and the supernatant was used for ESI-MS and ESI-MS/MS analyses.

A blank to disclose the possible release of compounds from the dialysis membrane was performed by dialysis of 100 mL of distilled water in 1 L of water during 48 h, with 1 L water exchange. The dialysate was eluted through a C_{18} column, and the retained material was washed with ultrapure water and recovered with acidic methanol (1.0% v/v acetic acid) in the same conditions as used for the sparkling wine foam. Solvents used were of HPLC grade.

Wine Model Solutions. Wine model solutions were constructed from a hydroalcoholic base solution with 10% ethanol (v/v) and 0.5% tartaric acid (w/v) adjusted at pH 3.5 with NaOH solution.[23,24] Glycerol and ethyl octanoate were also added to attain concentrations of 0.7% (w/v)[16,25] and 0.4% (w/v),[26] respectively. The fractions obtained from the sparkling wine FP_{LC} were added individually and in combination to the wine model solution for measurement of their foam properties.

Foam Property Measurement. Foamability and foam stability were assessed using an adaptation of the Mosalux and Bikerman method.[10,11,16] Analytical grade CO_2 from a cylinder flowed through a glass frit fitted in the bottom of a column (530 × 15 mm i.d.). The gas flow rate was controlled at 10 L/h by a flow meter (Cole-Parmer Instruments Co.). Foamability was evaluated as the increase in height of 10 mL of degassed sparkling wine or model wine solutions placed inside the glass column, after CO_2 injection through the glass frit. Two parameters of foamability were measured: (1) Maximum height reached by foam after CO_2 injection through the glass frit (HM, expressed in cm) represents the solution's ability to foam. (2) Foam stability height during CO_2 injection (HS, expressed in cm) represents the solution's ability to produce stable foam persistence of foam collar. Foam stability time (TS) was evaluated as the time elapsed before bubble collapse until the liquid appears after the interruption of CO_2 and is expressed in seconds. Each bottle of sparkling wine was analyzed in duplicate, and for each type of wine eight replicates (four bottles × two replicates per bottle) were obtained. The isolated fractions obtained from the wine were added independently or in mixtures to the wine model solution, taking into account their average proportions in these seven sparkling wines. For these solutions, the foam properties measurements were done with five replicates.

Chemical Analysis. *Sugar Analysis.* Monosaccharides were released from cell wall polysaccharides by a prehydrolysis in 0.2 mL of 72% H_2SO_4 (w/w) for 3 h at room temperature followed by 2.5 h of hydrolysis in 1 M H_2SO_4 at 100 °C. Neutral sugars were analyzed after conversion to their alditol acetates by GC, using 2-deoxyglucose as internal standard.[27,28] A Perkin-Elmer Clarus 400 GC apparatus with split injector and a FID detector was used, equipped with a 30 m column DB-225 (J&W) with i.d. and film thickness of 0.25 mm and 0.15 μm, respectively. The oven temperature program used was as follows: initial temperature, 200 °C, a rise in temperature at a rate of 40 °C/min until 220 °C, and then 220 °C for 7 min, followed by an increase until 230 °C at a rate of 20 °C/min, this temperature being maintained for 1 min. The injector and detector temperatures were, respectively, 220 and 230 °C. The flow rate of the carrier gas (H_2) was set at 1 mL/min. Uronic acids (UA) were quantified by a modification[27] of the 3-phenylphenol colorimetric method.[29] Sugar analysis was assayed for the HMW of the seven sparkling wines and for AqIMW, MeIMW, and MeLMW of FP_{LC} sparkling wine.

Protein Analysis. Protein quantification was based on the bicinchoninic acid (BCA) method using bovine serum albumin (BSA) as standard, using the Bicinchoninic Acid Protein Assay Kit from Sigma (Aldrich-Chemie, Steinheim, Germany).[23] Protein analysis was assayed for AqIMW, MeIMW, and MeLMW of FP_{LC} sparkling wine.

Amino Acid Analysis. Amino acid quantification was performed for the hydrophobic low molecular weight fraction <1 kDa (MeLMW) and for the most acidic subfraction of that, obtained from normal phase purification (F3). The amino acid residues were released by acidic hydrolysis,[30] derivatized with heptafluorobutyric anhydride, and the *N*-heptafluorobutyryl isobutyl esters of amino acids were analyzed by GC-FID.[31,32] Calibration curves for Ala, Val, Leu, Asx, and Glx were obtained in the concentration range of 0.0−0.2 mg/mL; for all other amino acids, the concentration range was 0.000−0.025 mg/mL.

Determination of Total Phenolic Compounds. Total phenolic composition was determined by the Folin−Ciocalteu colorimetric method,[23,33] using gallic acid as standard. The analysis of total phenolic compounds was performed for AqIMW, MeIMW, and MeLMW of FP_{LC} sparkling wine.

Electrospray Ionization Mass Spectrometry Conditions. ESI-MS analyses were performed on the subfraction of MeLMW recovery with MeOH acidic from silica gel column (F3) and on the hydrophobic low molecular weight material obtained from sparkling wine foam. Prior to MS analysis, sample F3 was dissolved in water and eluted through a C_{18} column, washed with diethyl ether, and recovered with MeOH (HPLC grade, Fisher Scientific, U.K.) with 1.0% (v/v) acetic acid. Both samples were independently concentrated and suspended in ultrapure water, and each solution (2 μL) was further diluted 100-fold in a MeOH/H_2O (1:1, v/v) solution with 1.0% (v/v) formic acid. The samples were introduced into the mass spectrometer using a flow rate of 8 μL/min. Positive ion mode ESI-MS and MS/MS spectra were acquired in an LXQ linear ion trap mass spectrometer (ThermoFinnigan, San Jose, CA). Typical ESI conditions were as follows: electrospray voltage, 5.0 kV; capillary temperature, 275 °C; sheath gas flow, 25 units. An isolation width of 0.5 Da was used with a 30 ms activation time for MS/MS experiments. Full scan MS spectra and MS/MS spectra were acquired with 50 and 200 ms maximum ionization times, respectively. Normalized Collision Energy (CE) was varied between 15 and 35 (arbitrary units) for both MS^2 and MS^3 according to the ion of interest. Data acquisition was carried out on an Xcalibur data system (V2.0). The water was of Milli-Q purity filtered through a 0.22 μm filter (Millipore, USA), and all organic solvents were of HPLC grade.

RESULTS AND DISCUSSION

Evaluation of Foam Aptitude of Bairrada Sparkling Wines. Seven sparkling wines were produced from two grape varieties (Fernão-Pires and Baga) using grapes from different ripening stages and soils. To evaluate the range of their foam aptitudes, the maximum height reached by foam after CO_2 injection through the glass frit, expressed in centimeters (HM), the foam stability height during CO_2 injection, also expressed in centimeters (HS), and the foam stability time, expressed in seconds (TS), were

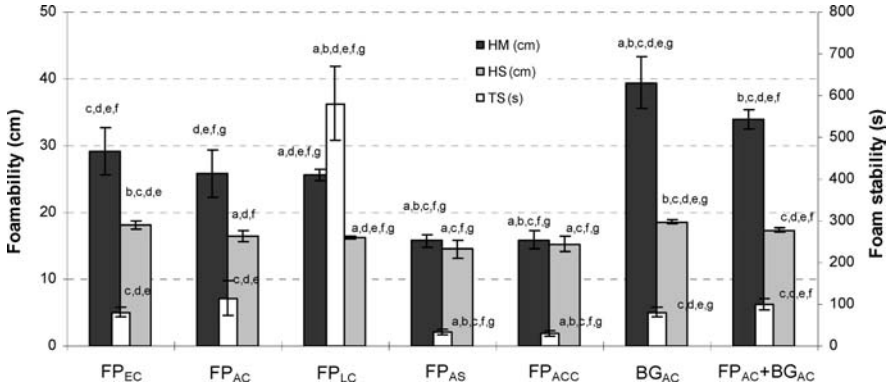


Figure 1. Foamability HM (maximum height reached by foam after CO_2 injection) and HS (foam stability height during CO_2 injection) and stability TS (foam stability time) measured for seven different sparkling wines from Bairrada appellation. Key: a, significantly different ($p < 0.05$) from FP $_{EC}$; b, significantly different ($p < 0.05$) from FP $_{AC}$; c, significantly different ($p < 0.05$) from FP $_{LC}$; d, significantly different ($p < 0.05$) from FP $_{AS}$; e, significantly different ($p < 0.05$) from FP $_{ACC}$; f, significantly different ($p < 0.05$) from BG $_{AC}$; g, significantly different ($p < 0.05$) from FP $_{AC}$+BG $_{AC}$.

measured (Figure 1). The HM ranged from 15.8 to 39.4 cm; the minimum values were observed for FP variety from grapes harvested at the adequate maturity, grown in sandy (FP_{AS}) and clay−calcareous (FP_{ACC}) soils, and the maximum was achieved for BG variety from grapes harvested at adequate maturity, grown in clayey soil (BG_{AC}). For HS, these sparkling wines showed a shorter interval than for HM, from 14.5 to 18.6 cm. The maximum and minimum HS were observed for the same samples as for HM. The range observed for TS varied between minima of 31 and 33 s, for FP_{AS} and FP_{ACC}, as observed for the other foam parameters, and a maximum of 582 s, observed for FP variety from grapes from a late harvest, grown in a clayey soil (FP_{LC}). However, the TS observed for FP_{LC} is much higher than the TS observed for all other sparkling wines. The range observed for TS without the contribution of this wine is 31−115 s. These results show that the foam aptitude of different Bairrada sparkling wines can vary considerably, mainly for foam stability time. Although TS seems to be influenced by the soil type (31 s for FP_{ACC}, from a clay−calcareous soil, 33 s for FP_{AS}, from a sandy soil, and 115 s for a clayey soil), the influence of the ripening stage of the grape at harvest showed the highest influence in clayey soil for the FP variety (81 s for early harvest, FP_{EC}, 115 s for harvest at maturity, FP_{AC}, and 582 s for late harvest, FP_{LC}). Considering the variety, the wines containing BG grapes showed highest HM values, but this characteristic is not present in HS or TS. These results are in accordance with the works carried out on sparkling wines from other appellations where the aptitude of some varieties to foamability and foam stability, as well as the impact on foam of harvest and winemaking process, was studied.[12,34,35]

Fractionation of Sparkling Wine Components and Relationship with Foam Properties. To observe the compounds present in sparkling wine that most influence their foam aptitude, the wines were fractionated according to the molecular weight of their components in high molecular weight (HMW) material, for example, compounds with molecular weight higher than 12 kDa, material with intermediate molecular weight (IMW), for example, compounds that diffused through the pores of the dialysis membrane of 12 kDa but were retained by the dialysis membrane of 1 kDa pores, and material with low molecular weight (LMW), for example, compounds that diffused through the pores of the dialysis membrane of 1 kDa. The IMW fraction was further divided according to its polarity into a hydrophobic fraction, extracted with acidic methanol from a C_{18} resin (MeIMW), and a hydrophilic one, not retained (AqIMW). The hydrophobic compounds were also recovered from the LMW fraction by extraction with acidic methanol from a C_{18} resin, giving rise to fraction MeLMW (Scheme 1).

Table 1 shows the yield of the four fractions obtained from each of the seven sparkling wines under study. The wines with lower TS, FP_{AS} and FP_{ACC}, were those with the lower amounts of MeLMW, 11.9 and 19.5 g/L, respectively. These values are 30- and 18-fold less, respectively, than that of FP_{LC}, the wine that has shown the highest TS. FP_{LC} wine also showed the higher yield in AqIMW. For the FP variety grown in clayey soil, the amount of UA present in wines decreased during ripening, from 16 to 11 to 3 mol % for early, adequate, and late harvest, respectively. The other sparkling wines (FP_{AS}, FP_{ACC}, and BG_{AC}) showed values between 10 and 12%, corresponding to wines produced with grapes picked up at the adequate harvest moment. This shows that the decrease in polymeric UA in wines seems to be related with the increase of TS value in wines.

The decrease in UA is related to the degradation of pectic polysaccharides with ripening, which is in accordance with Yakushiji et al.,[36] who reported degradation of cell-wall polysaccharides from the mesocarp of grape berries when comparing *véraison* with maturity. Although the acidic polysaccharides did not show any correlation with foamability,[7] galacturonic acid content was anticorrelated with TS by Andrés-Lacueva et al.,[12] showing that the wines with the lowest galacturonic content had better TS. The degradation of pectic polysaccharides observed for the late harvest results in the decrease of UA in the HMW fraction with its consequent increase in the AqIMW, as shown in Table 2.

Many factors have been correlated with foam properties, namely, *Botrytis cinerea*, wine aging, and bentonite addition. *B. cinerea* infection has a negative influence on foam properties;[37] wine aging for 18 months was reported to confer the best HM and TS, apparently due to the release of proteins and polysaccharides by yeast autolysis,[38] and bentonite addition was reported to promote a decrease of HS and TS, possibly due to the reduction of total soluble protein concentration.[39] These three studies related foam properties with wine composition as modulated by treatments. To understand the influence of the different wine components and their possible synergistic effects in foam behavior, reconstituted sparkling wine solutions were prepared from the HMW, MeIMW, AqIMW, and MeLMW fractions obtained from FP_{LC}, the sparkling wine that presented the highest TS.

Table 1. Sugar Composition of HMW and Yields of HMW, MeLMW, MeIMW, and AqIMW from Sparkling Wine

	yield (mg/L)				mol %								
wine	MeLMW	MeIMW	AqIMW	HMW[a]	Rha	Fuc	Ara	Xyl	Man	Gal	Glc	UA	total sugars (%, w/w)
FP_{EC}	532.9	25.6	6.4	349.6 (8)	2	0	9	0	39	29	4	16	66
FP_{AC}	412.3	28.5	7.9	448.0 (21)	2	0	10	0	45	24	7	11	63
FP_{LC}	359.5	26.3	19.9	422.8 (5)	2	0	11	1	43	32	8	3	46
FP_{AS}	11.9	1.7	4.7	352.4 (2)	2	1	11	0	39	28	6	12	73
FP_{ACC}	19.5	0.8	7.0	480.8 (3)	4	0	11	0	46	26	3	10	70
BG_{AC}	47.0	1.1	8.1	417.5 (8)	2	0	13	0	39	27	7	11	54
FP_{AC}+BG_{AC}	124.3	0.5	7.2	550.6 (3)	2	0	13	0	37	30	8	10	51

[a] Average of four independent extractions, with the exception of the wine FP_{AC} + BG_{AC} that has two replicates (%RSD in parentheses).

Table 2. Sugar Composition and Total Sugar, Total Protein, and Total Phenolic Contents of the Intermediate and Low Molecular Weight Fractions Isolated from Sparkling Wine (FP_{LC})

	mol %										
fraction	Rha	Fuc	Ara	Xyl	Man	Gal	Glc	UA	total sugars (%, w/w)	protein (%, w/w)	phenolic compounds (%, w/w)
AqIMW	7	1	6	3	30	11	10	32	53	19	18
MeIMW	3	1	15	6	10	4	45	18	9	*a*	*a*
MeLMW	2	1	10	5	20	4	49	10	7	39	6

[a] Fraction with high content of protein and phenolic compounds.

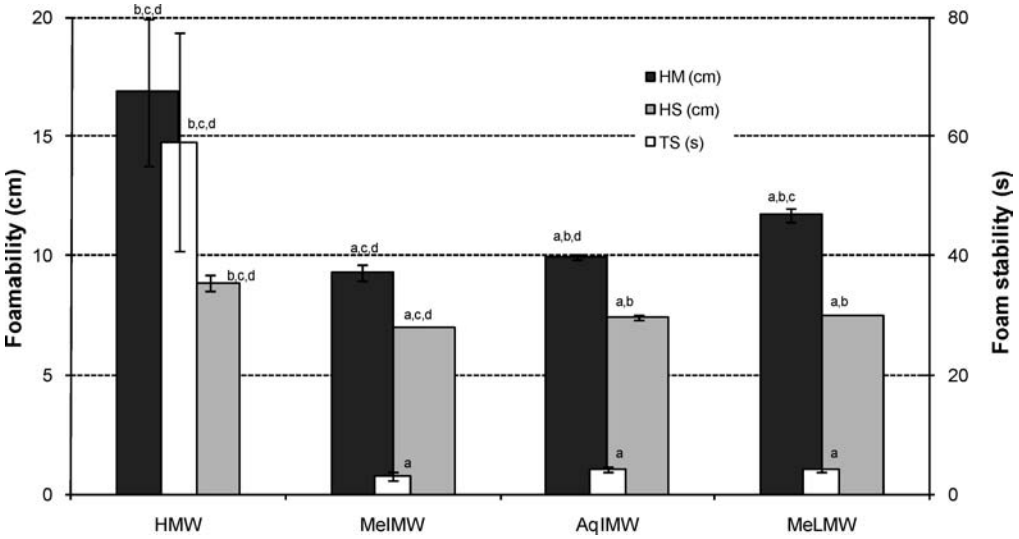


Figure 2. Foamability, HM and HS, and foam stability, TS, measured for the fractions previously isolated from wine (FP_{LC}). Key: a, significantly different ($p < 0.05$) from HMW; b, significantly different ($p < 0.05$) from MeIMW; c, significantly different ($p < 0.05$) from AqIMW; d, significantly different ($p < 0.05$) from MeLMW. All fractions were in wine concentration in the model solution.

Evaluation of Foam Properties of Individual Sparkling Wine Fractions in Model Solutions. Figure 2 shows the foam evaluation of the wine model solutions reconstituted from each of the four fractions previously obtained from FP_{LC} sparkling wine (Scheme 1). For the reconstitution, the same amount of material recovered from the wine was used (Table 1): 420 mg/L for HWM, 26 mg/L for MeIWM, 20 mg/L for AqIWM, and 360 mg/L for MeLWM.

The better foam properties, for example, the higher HM, HS, and TS, were observed for HMW. The HM increased in the sequence MeIMW (9.3 cm), AqIMW (10.0 cm), MeLMW (11.7 cm), and HMW (16.9 cm). For the HS, a slight variation between 7.0 and 8.9 cm was observed for MeIMW and HMW, respectively. Furthermore, TS showed values 14−20-fold higher than for the other fractions (59 s for HWM and 3−4 s for the others). These foam measurements showed that the wine model solutions reconstituted with the HMW fraction, for itself, explained 65, 53, and 9% of HM, HS, and TS, respectively, of the foam values achieved for the FP_{LC} sparkling wine (Figures 1 and 2). This HMW fraction was composed by 46% of sugars (Table 1), with a sugar composition mainly constituted by Man (43 mol %), Gal (32 mol %), and Ara (11 mol %). According to the bibliography, these sugar residues are components of mannoproteins from yeast and arabinogalactans and pectic polysaccharides from

grapes.[40,41] As the HWMs from the different wines have similar contents and compositions, this fraction, by itself, does not explain the differences in foam properties of these wines. To observe the possible presence of a synergistic effect between the components of the different fractions, the foam evaluation of wine model solutions containing combined fractions was performed.

Evaluation of Foam Properties of Model Solutions of Combined Wine Fractions. The foam parameters of the simultaneous combination of the four fractions, 420 mg of HMW + 26 mg of MeIMW + 20 mg of AqIMW + 360 mg of MeLMW in 1 L of 10% alcoholic solution, simulating a total reconstitution of original sparkling wine, when compared with the solution containing the HMW fraction, had similar HM (15.3 cm) and HS (8.6 cm) but considerably lower TS (6 s). The total wine reconstitution represented only 1% of the TS achieved for the sparkling wine. It is possible that the mixture of these different molecules has different contributions to the foam aptitude, as some of them could have a positive and others a negative effect on foam. In fact, according to Table 2, the fraction AqIMW was composed mainly by sugars (53%) followed by proteins (19%) and phenolic compounds (18%). The sugar composition showed 32 mol % of UA and 30 mol % of Man, sugars that are characteristic of pectic polysaccharides and mannoproteins, respectively.[40,41] On the other hand, the fraction MeIMW was composed mostly by phenolic compounds and proteins. The colorimetric methods used for quantification of phenolic compounds and proteins present mutual interferences of these compounds, preventing their realistic quantification. The fraction MeLMW showed to be constituted mainly by peptides (39%), followed by sugars (7%) and phenolic compounds (6%). The major sugar was glucose, possibly arising from the glycosylation of phenolic compounds.[42,43] The amino acid composition of fraction MeLMW (Table 3) showed that the major amino acid was Glx (estimated by the sum of Glu and Gln) at 13.12 μg/mg, followed by Asx (estimated by the sum of Asp and Asn), Leu, and Gly at 9.79, 9.03, and 8.59, respectively. The amino acid profile in the free form was quite similar to the total amino acid content profile, with the exception of Pro, which was the third major amino acid instead of Leu (Table 3).

When the HMW was combined with the MeLMW fraction in the proportions recovered from the sparkling wine (420 and 360 mg/L), a wine model solution was obtained presenting HM and HS of 16.3 and 8.6 cm, values that are similar to those observed for the solution containing the HMW fraction alone (Figures 2 and 3). This combination also showed a TS of 161 s, a value 2.7 times higher than that obtained for the TS of the HMW fraction alone (59 s). This value of TS showed that the wine model solution reconstituted with the HMW + MeLMW fractions explained 24% of the TS measured for the FP_{LC} sparkling wine.

Evaluation of Foam Properties of the Combination of HMW with Subfractions of MeLMW Material (F1−F3). To better understand the foam behavior in relation to the solution composition containing the HMW fraction and the low molecular weight hydrophobic material present in the fraction MeLMW, the latter material was further fractioned. The MeLMW material was separated by polarity through a silica gel column into three fractions: F1 was the most hydrophobic fraction, F2 had an intermediate hydrophobicity, and F3 was the least hydrophobic. These three fractions were individually added to the model wine solutions containing the HMW material. F1, F2, and F3 were added in the amount recovered from the wine, for example, 90, 37, and 228 mg/L, respectively, and the foam aptitude of the resultant solutions was measured (Figure 3). For all solutions, the HM, HS, and TS measured were significantly higher than for the HMW + MeLMW. The TS value also increased in the order HMW + F1, HMW + F2, and HMW + F3, from the highest hydrophobic material to the least hydrophobic one. No significant differences were observed for HM, HS, and TS between HMW + F2 and HMW + F3, but the latter showed better relative standard deviation, namely, in TS. The foam range values observed for the addition of these three subfractions to the HMW were close to the values observed for the wine, as HM represents 77−85% of sparkling wine HM, HS represents 59% of sparkling wine HS, and TS represents 34−68% of sparkling wine TS. Furthermore, the fractions HMW, HMW + MeLMW, HMW + F1, HMW + F2, and HMW + F3 showed HM and TS in the range of values observed for the sparkling wines, as only HS (8.6−10.0 cm) was under the interval (14.5−18.6 cm). Subfraction F3 seems to be an important fraction to explain the foam behavior, as its presence in solution together with HMW allowed an increase of 8-fold in TS.

Table 3. Total and Free Amino Acid Compositions of Fraction MeLMW and Total Amino Acid Content of F3 (the Most Acidic Subfraction Obtained from MeLMW in the Normal Phase Column)

	concentration (μg/mg)		
	MeLMW		F3
amino acid	total	free	total
Ala	1.68	0.37	0.07
Gly	8.59	2.40	0.43
Val	6.92	0.46	0.29
Thr	2.37	0.36	0.10
Ser	2.04	0.57	0.08
Leu	9.03	1.73	0.30
Ile	7.12	0.49	0.25
Pro	6.79	2.99	0.23
Hyp	1.72	0.63	0.30
Asx	9.79	7.23	0.08
Phe	2.40	0.61	
Glx	13.12	5.03	0.45
Lys	2.56	0.48	
Tyr	2.03	0.57	
Arg	tr[a]	tr	
total	74.65	23.46	2.16

[a] tr, trace amounts.

On the basis of the assumption that the stability of Champagne bubbles requires the presence of an adsorption layer, a recent study was described by Abdallah et al.[44] to evaluate the hypothesis of the significant contribution of macromolecules to the formation of the adsorption layer at the interface with the gases. These authors studied three macromolecular fractions (>100, >30, and >10 kDa) isolated from native Champagne wines. The isolated macromolecules were dissolved in a wine matrix constituted by an ultrafiltered wine submitted to a cutoff of 5 kDa, and the surface activity was measured by ellipsometry. This study showed that the macromolecules present in Champagne allowed the formation of the adsorption layers comparable to those observed at the surface of native wines.[44] In fact, this study corroborates our findings showing that the use of ultrafiltered wine with a cutoff 5 kDa as wine model solution in combination with the

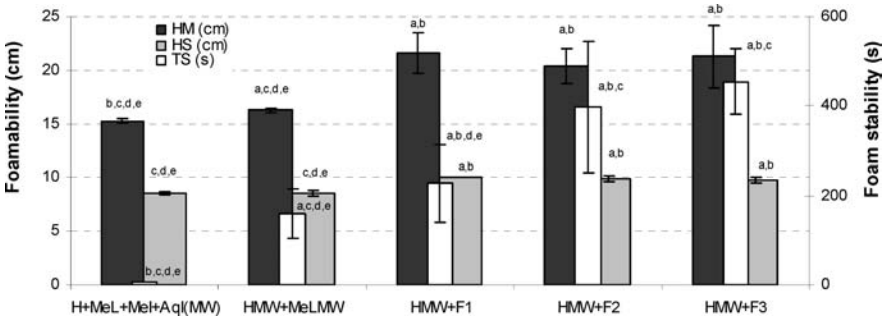
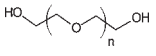


Figure 3. Foamability, HM and HS, and foam stability, TS, measured for reconstituted wine (FP_{LC}): total wine reconstitution (H+MeL+MeI+AqI(MW)), HMW+MeLMW fraction, and HMW plus the three fractions isolated from MeLMW by a silica gel column (F1, F2, and F3). All fractions present in the model solution were added in the concentration found in wine. Key: a, significantly different ($p < 0.05$) from HMW+MeLMW+MeIMW+AqIMW; b, significantly different ($p < 0.05$) from HMW+MeLMW; c, significantly different ($p < 0.05$) from HMW+F1; d, significantly different ($p < 0.05$) from HMW+F2; e, significantly different ($p < 0.05$) from HMW+F3.

wine high molecular weight fractions allowed reconstituting the sparkling wine foam properties.

The literature available regarding the relationship of wine low molecular weight molecules and foam properties proposes peptides of low molecular weight (200−300 Da) as foam stabilizers.[13] The presence of aromatic amino acids (that confer hydrophobicity to the peptides) in Cava sparkling wines has also been shown to improve the quality of the foam of these wines.[14] Table 3 shows the amino acid content of fraction F3. The major amino acids were Glx (0.45 μg/mg) and Gly (0.43 μg/mg), in a total concentration of amino acids of 2.16 μg/mg, which does not explain the chemical composition of this fraction. The sugar analysis was also assessed, showing only 26 μg/mg; the major sugars were Glc (42 mol %) and UA (34 mol %). To assess a detailed composition of F3 that could explain its relevant foam properties, it was analyzed by ESI-MS and MS/MS.

ESI-MS and ESI-MS/MS Characterization of Fraction F3. Figure 4a shows the ESI-MS spectrum of fraction F3. The ions at m/z 305, 349, 393, 437, 481, 525, and 569 show differences of 44 Da. According to the ESI-MS/MS spectra of these ions, for each ion were observed successive neutral losses of 44 Da (data not shown), thus making it possible to assign them to the sodiated adducts of polyethylene glycol ($OH-CH_2-(CH_2-O-CH_2)_n-CH_2-OH$; see structure below), where n varies from 5 to 11.



These molecules could have natural or technological origin. The presence of ethylene glycol in wines has been reported as a native constituent,[45,46] produced by yeast from ethanolamine via glycolaldehyde. The strain *Zygosaccharomyces bailii* 429 (a yeast species that is also found in wine) has been reported as the major ethylene glycol producer, accounting for more than half of the ethanolamine consumed. Under aerobic, as well as anaerobic, conditions, strains of *Saccharomyces cerevisiae* formed only small amounts of ethylene glycol.[46] Polyethylene glycol could also have a technological origin, as it is used in bioprocessing, promoting the increase of the release of extracellular products through interaction with cell membrane components during the fermentation step.[47] Also, it is also used to control fermentation foam.[2] Polyethylene glycol enhances the solubilization of surfactants,[48] the amphiphilic compounds that can reduce surface and interfacial tensions by accumulating at the interface of immiscible fluids,

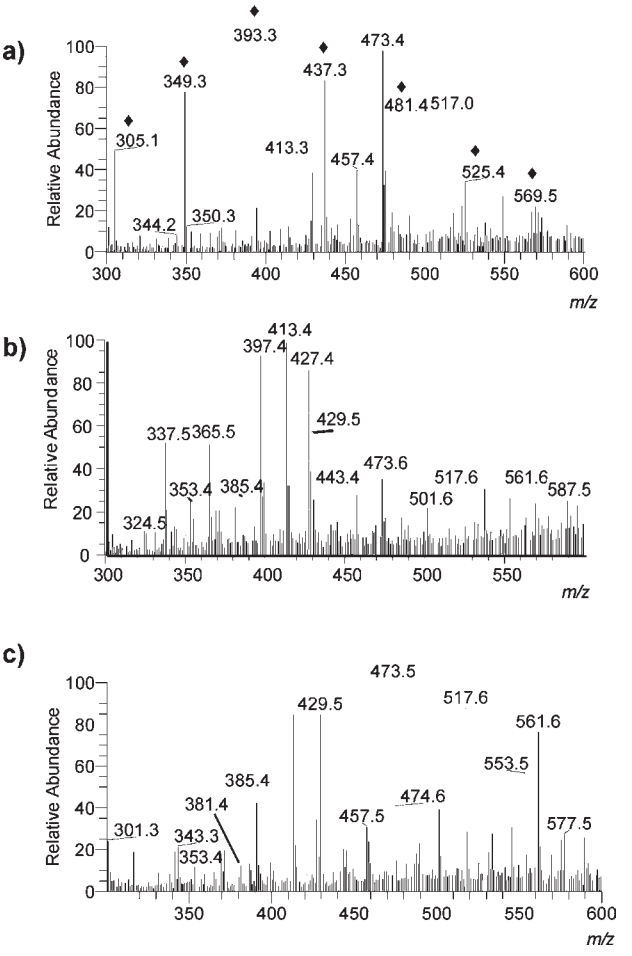


Figure 4. Mass spectrum of full MS acquisition by ESI-MS of (a) fraction F3 (diamonds (◆) indicate the polyethylene glycol series from $n = 5$ to $n = 11$), (b) low molecular weight hydrophobic compounds of sparkling wine foam after addition of lithium acetate, and (c) low molecular weight hydrophobic compounds of sparkling wine foam.

increasing the solubility, mobility, and bioavailability of immiscible components.[47] To our knowledge, and according to the wine producers, no additives have been used during the winemaking of the sparkling wines used in this study. A blank to disclose the possible release of compounds from the dialysis membranes, both 1 and 12 kDa, was performed, but none of these compounds were

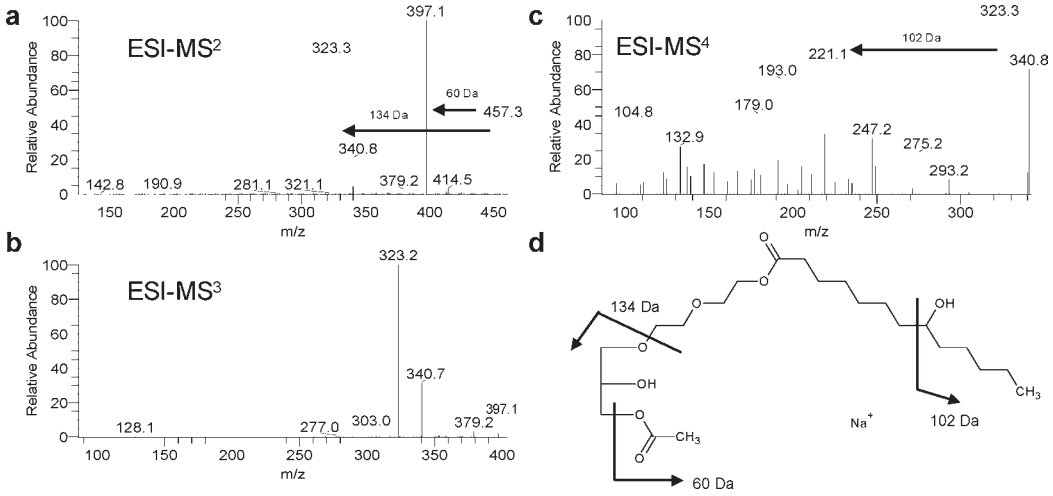


Figure 5. ESI-MS^n spectra of ion at m/z 457 present in fraction F3: (a) MS^2 of ion at m/z 457; (b) MS^3 of ion at m/z 397; (c) MS^4 of ion at m/z 323; (d) tentative structure assignment.

identified. The ion at m/z 413 is a contaminant, as it was also present in the spectrum of the solvent.

The ESI-MS/MS of the ion at m/z 457 (Figure 5a) showed the major neutral loss of 60 Da, attributed to an acetic acid molecule, with formation of the ion at m/z 397, and the loss of 134 Da attributed to the loss of a glycerol acetate molecule, with formation of the ion at m/z 323. The ion at m/z 397 showed also by MS^3 the formation of the ion at m/z 323 (Figure 5b), allowing the presence of a sodiated glyceryl derivative to be inferred. The MS^4 of the product ion at m/z 323 (Figure 5c) showed successive losses of neutral molecules with differences of 14 Da, characteristic of a carbon chain fragmentation profile.[49,50] The main neutral loss was 102 Da, which can be attributed to a hydroxylated carbon chain fatty acid, as shown in Figure 5d. The successive cleavages of the C−C bonds result in the neutral losses of 116, 130, 144, 158, and 172 Da, giving the ions at m/z 207, 193, 179, 165, and 151, respectively. This fragmentation pattern allows the structure shown in Figure 5d to be proposed for the glyceryl acetate diethylene glycol 8-hydroxytridecanoate, although the order of the substituents in the glycerol moiety is still uncertain. Ether-containing polar lipids are rare,[51] being mainly confined to the Archaea domain.[52,53] Anyway, ether-containing lipids were described to occur in alkylglycerols, namely, 1-*O*-alkyl/alkenyl-2-*O*-acyl-glycero-3-phosphocholine, found in the cell membrane of *Mycoplasma fermentans* [54], and also glycerol ethers sugar derivatives in *Propionibacterium propionicum*.[55] It is possible that the low molecular weight compounds found in these fractions have also a microbial origin.

ESI-MS Characterization of Hydrophobic Low Molecular Weight Material from Sparkling Wine Foam. To find potential tensioactive molecules that can be present in the low molecular weight fraction obtained from sparkling wine foam, the compounds present in the foam recovered by dialysis and reverse-phase chromatography were analyzed by ESI-MS. As soft ionization methods usually give different ions depending on the type of cations involved on the ionization procedure, ESI-MS analysis was performed using lithium and sodium adducts.

Figure 4b shows the full MS spectrum of the lithium ions of low molecular weight hydrophobic compounds obtained from sparkling wine foam. The most abundant ions were obtained at m/z 337, 365, 397, 413, 427, 429, 473, 517, and 561. As the ion at m/z 413 was also present in the spectrum of the solvent, it was assumed to be a contaminant. The ions at m/z 397, 427, 473, 517, and 561 were not yet possible to assign. Figure 4c shows the full MS spectrum of the sodium ions of low molecular weight hydrophobic compounds obtained from sparkling wine foam. The most abundant ions were obtained at m/z 343, 369, 385, 413, 429, 457, 473, 517, 553, and 561. As the ion at m/z 413 was also present in the spectrum of the solvent, it was assumed to be a contaminant. The ions at m/z 473, 515, 553, and 561 were not yet possible to assign, although the ions at m/z 473 and 561 are in common in both spectra (Figure 4, panels b and c). Tandem mass spectrometry (ESI-MS^n) was performed to identify the ions obtained.

Evidence of the Occurrence of Monoacyl Glycerols in Sparkling Wine Foam. To determine the structures of the ions occurring as lithium adducts in the ESI-MS spectrum, they were submitted to tandem MS analysis. The ion at m/z 337 showed a loss of 74 Da, attributed to a glyceryl moiety, giving the ion at m/z 263 (Figure 6b), and a loss of 238 Da that corresponds to the ketene form of palmitic acid ($C_{14}H_{29}$—CH=C=O), at m/z 99. This fragmentation allows the presence of [glyceryl palmitate + Li]$^+$ to be inferred. The ion at m/z 365 showed also the loss of 74 Da attributed to a glyceryl moiety, giving the ion at m/z 291 (Figure 6c), allowing the presence of [glyceryl stearate + Li]$^+$ to be inferred. These fragmentation patterns allowed assignment of these two ions to two potential tensioactive molecules, glyceryl palmitate and glyceryl stearate, present in this sparkling wine foam. Monoacylglycerol of fatty acids (C_{14}−C_{18}) belong to the food emulsifiers (E471 series) class. Indeed, they improve the manufacture of products by acting as foam and cream stabilizers, crumb softeners, or staling agent inhibitors.[56] Studies on maternal milk have demonstrated that monoacylglycerols also exhibit antibacterial and antiviral properties.[57] In addition, glyceryl palmitate is a cosmetic ingredient used as an emollient and/or surfactant-emulsifying agent.[58] With regard to these compounds in sparkling wine foam, no reports are yet available. Anyway, free fatty acids and their ethyl esters are known compounds of base wine-induced foam[4] and have also been reported to be present in aerosols released by the collapsed bubbles of Champagne wine.[22] Monoacylglycerols have been reported to be released into the wine by yeast autolysis.[9] The release of fatty acids from hydrolysis of the monoacylglycerols from Champagne wines showed the presence of the fatty acids 16:0, 16:1, 18:0, and 18:1 and one

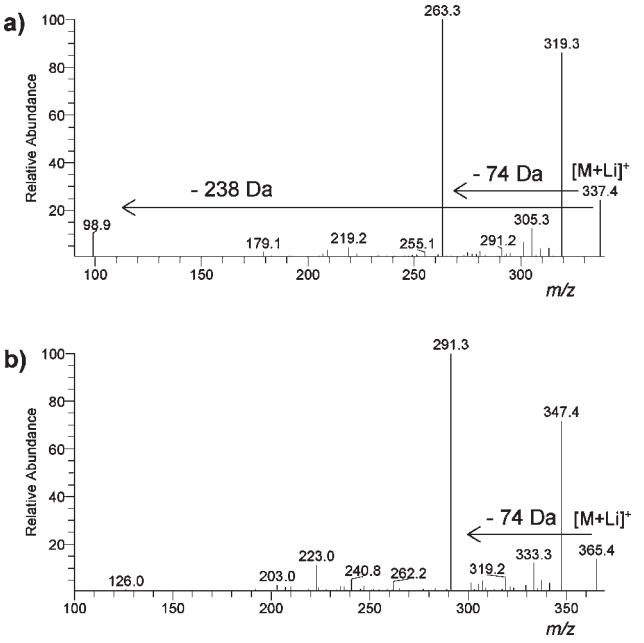


Figure 6. ESI-MS^2 spectra of lithium adducts of low molecular weight hydrophobic compounds of sparkling wine foam, $[M + Li]^+$ ions at (a) m/z 337 and (b) m/z 365.

oxidized fatty acid.[59] Although in small relative abundance, the sodium adducts of glyceryl palmitate and glyceryl stearate can also be observed at m/z 353 and 381 in Figure 4c (fragmentation data not shown).

Evidence of the Occurrence of Glycerylethylene Glycol Fatty Acid Derivatives in Sparkling Wine Foam. To observe if other surface-active molecules can be present as sodium adducts in the sample of sparkling wine foam, all major ions present in the ESI-MS spectrum of low molecular weight hydrophobic compounds obtained from sparkling wine foam were studied by tandem MS. The ions at m/z 369, 385, 429, and 457 exhibit fragment ions that are consistent with the presence of glyceryl fatty acid derivatives. For the ion at m/z 369, the major ion was formed at m/z 324, which can be attributed to the loss of a formic acid radical ($HCOOH^{\cdot}$, Figure 7a). Also, the MS^2 spectrum shows the ion at m/z 251, the result of a loss of 118 Da, attributed to the loss of glycerylformate. The MS^3 spectrum ion at m/z 324 shows the ion at m/z 97, attributed to the sodiated glyceryl residue, confirming the occurrence of a glyceryl moiety in this molecule and allowing the occurrence of an esterification of glycerol by a formic acid to be inferred. This product ion spectrum also shows the ions at m/z 137, 123, 109, and 95, resulting from successive losses with differences of 14 Da, consistent with a saturated hydrocarbon chain fragmentation profile.[49,50] The loss of 184 Da from the ion at m/z 369 observed in the MS^2 spectrum can be attributed to a dodecanoic acid residue. The ion at m/z 369 can be attributed to a glycerylformate associated with a dodecanoic acid moiety by a 44 Da linker, possibly a monoethylene glycol residue. The MS^2 spectrum also shows the ion at m/z 185, attributed to [glycerylformate monoethylene glycol + Na]$^+$ and the ion at m/z 267, MS^3 (324 → 267), showing the loss of 56 Da (glyceryl residue − H_2O), attributed to [monoethylene glycol dodecanoate + Na]$^+$. On the basis of these results, one possible assignment for the ion at m/z 369 was sodiated glycerylformate monoethylene glycol dodecanoate. The ESI-MS spectrum of the blank sample showed the occurrence of a low-intensity ion at m/z 369.3. However, its fragmentation resulted in a very different pattern of different fragment ions (results not shown), allowing the conclusion that this ion is not an artifact of the methodology used.

The ion at m/z 385 gave a MS^2 spectrum with the main fragments at m/z 367, 324, 281, and 213, due to the neutral loss of 18, 61, 104, and 172 Da, corresponding to the loss of H_2O, acetate radical ($CH_3COOH^{\cdot}$), $C_4H_8O_3$, and $C_{10}H_{20}O_2$, respectively (Figure 7b). The MS^3 of the ion at m/z 324 showed several product ions consistent with a saturated hydrocarbon chain fragmentation, with cleavages at C_β, C_γ, and C_δ, leading to formation of the ions at m/z 123, 109, and 95, respectively. On the basis of these results, the ion at m/z 385 can be assigned to the sodiated glycerylacetate diethylene glycol nonanoate ion. The ESI-MS spectrum of the blank sample showed the occurrence of a very low intensity ion at m/z 385.1. Its fragmentation resulted in a very different pattern of different fragment ions (results not shown), allowing the conclusion that it is not an artifact of the methodology used.

The ion at m/z 429 showed a MS^2 spectrum with a fragmentation pattern similar to that of the ion at m/z 457 (Figure 7c,d). In both spectra, the major fragment ion neutral losses correspond to 60 Da, attributed to an acetic acid molecule, with the formation of the ions at m/z 369 and 397, respectively, and loss of 134 Da, attributed to a glycerylacetate molecule, with formation of the ions at m/z 295 and 323, respectively. The MS^3 spectrum of the ion at m/z 295 (Figure 7d) showed the ions at m/z 193, 179, 165, 151, and 137, resulting from successive losses with differences of 14 Da, consistent with a saturated hydrocarbon chain fragmentation profile. The most intense fragment was the ion at m/z 193, with loss of 102 Da that can be attributed to a hydroxylated carbon chain fatty acid, as shown in Figure 7c. On the basis of these results and the fragmentation consistent with the presence of ethylene glycol in previous structures, the ion at m/z 429 can be assigned to the sodium adduct of glycerylacetate diethylene glycol-6-hydroxyundecanoate. The MS^3 spectrum of the ion at m/z 323, from the parent ion at m/z 457, showed the ions at m/z 221, 207, 193, 179, 165, and 151. These ions and fragmentation profile are similar to those observed for the ion at m/z 457 of F3 fraction (Figures 5a,c and 7d). On the basis of these results, the ion at m/z 457 can be assigned as the sodium adduct of glycerylacetate diethylene glycol 8-hydroxytridecanoate (Figure 7d). The ESI-MS spectrum of the blank sample showed the occurrence of ions at m/z 429.3 and 457.3. Their fragmentation resulted in a very different pattern of different fragment ions (results not shown), allowing the conclusion that they are not artifacts of the methodology used.

As observed for monoacylglycerols, the glycerylethylene glycol fatty acyl derivatives here reported to be present in sparkling wine foam have potentially surfactant properties due to their more hydrophilic (glyceryl moiety) and more hydrophobic (fatty acid residue) components. The compounds identified have a structure similar to that of the synthetic polyethoxylated nonionic surfactants, glycerol polyoxyethylene (POE) ricinoleates, which are composed by glycerol tripolyethylene glycol ethers ($n = 12-38$) esterified by one, two, or three molecules of ricinoleic acid.[60]

In summary, the data obtained allowed the conclusion that sparkling wine foam presented glycerylethylene glycol fatty acid derivatives. These compounds have been shown to be involved in foam promotion and stabilization of wine model solutions. A higher number of glycerylethylene glycol fatty acid derivatives was found in sparkling wine foam than in the fraction containing the low molecular weight hydrophobic material recovered from the whole sparkling wine (F3). As the same sparkling wine

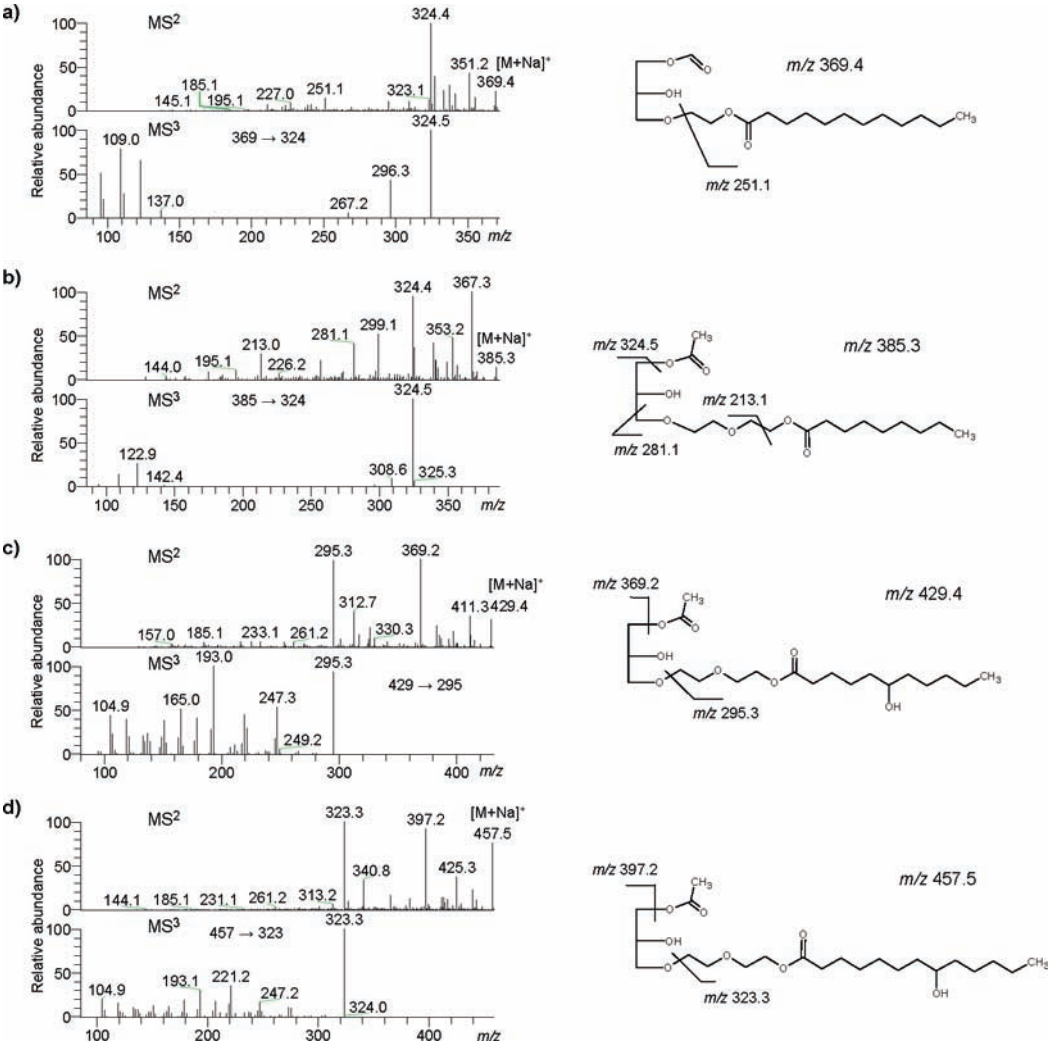


Figure 7. MS^2 and MS^3 spectra fragmentation of sodium adducts of ions at (a) m/z 369, (b) m/z 385, (c) m/z 429, and (d) m/z 457 and tentative structure assignments for these ions, present in sparkling wine foam.

(FP_{LC}) was used to obtain the two samples, it is possible to infer that these surface active compounds are preferentially partitioned by the sparkling wine foam rather than the liquid phase, as observed for Champagne aerosols and bulk by Liger-Belair et al.[22] In addition to glycerylethylene glycol fatty acid derivatives, the monoacylglycerols are also surface active compounds present in sparkling wine foam. These results showed that the combination of high and low molecular weight molecules promotes a synergistic effect in foamability and foam stability of sparkling wines.

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ABBREVIATIONS USED

AqIMW, aqueous intermediate molecular weight material; BG_{AC}, sparkling wine produced with Baga grape variety from an adequate harvest maturity from a clayey soil; F1, fraction 1, the more hydrophobic subfraction, obtained from the hydrophobic low molecular weight material retained in a silica gel column and eluted with dichloromethane/methanol (1:1, v/v); F2, fraction 2, the intermediate hydrophobic subfraction, obtained from the hydrophobic low molecular weight material retained in a silica gel column and eluted with methanol; F3, fraction 3, the less hydrophobic subfraction, obtained from the hydrophobic low molecular weight material retained in a silica gel column and eluted with acidic methanol; FP_{AC}, sparkling wine produced with Fernão-Pires grape variety from an adequate harvest maturity from a clayey soil; FP_{AC}+BG_{AC}, sparkling wine produced with a mixture of must (50:50) Fernão-Pires and Baga grape varieties from an adequate harvest maturity from a clayey soil; FP_{ACC}, sparkling wine produced with Fernão-Pires grape variety from an adequate harvest maturity from a clay−calcareous soil; FP_{AS}, sparkling wine produced with Fernão-Pires grape variety from an adequate harvest maturity from a sandy soil; FP_{EC}, sparkling wine produced with Fernão-Pires grape variety from an early harvest moment (1 week before maturity)

from a clayey soil; FP_{LC}, sparkling wine produced with Fernão-Pires grape variety from a late harvest moment (1 week after maturity) from a clayey soil; HMW, high molecular weight material, >12 kDa; IMW, intermediate molecular weight material, between 1 and 12 kDa; LMW, low molecular weight material, <1 kDa; MeIMW, hydrophobic intermediate molecular weight material; MeLMW, hydrophobic low molecular weight material.

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