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The influence of plant- and yeast-derived proteins on gushing in sparkling wine

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Abbreviations

%	percentage
× *g*	times gravity
µm	micrometer
ACN	acetonitrile
APS	ammonium persulphate
B. cinerea	*Botrytis cinerea*
BCIP	5-bromo-4-chloro-3-indolyl phosphate
BLAST	Basic Local Alignment Search Tool
BSA	albumin fraction V
cm	centimeter
CO_2	carbon dioxide
ddH_2O	double distilled water
dH_2O	demineralized water
DMF	dimethylformamide
DTT	dithiothreitol
EDTA	ethylendiaminetetra-acetic acid
ELISA	enzyme-linked immunosorbent assay
FPLC	fast protein liquid chromatography
h	hour
H. vulgare	*Hordeum vulgare*
H_2CO_3	carbonic acid
HCCA	α-Cyano-4-hydroxycinnamic acid
HCl	hydrogen chloride
hl	hectoliter
K_2HPO_4	dipotassium hydrogen phosphate
KCl	potassium chloride

kDa	kilodalton
kg	kilogram
L	liter
M	molarity
mA	milliampere
MALDI-TOF-MS	matrix-assisted desorption/ionization time-of-flight mass spectrometry
mAU	milli-absorbance units
$MgCl_2$	magnesium chloride
min	minute
ml	milliliter
mm	millimeter
mmol	millimole
Na_2HPO_4	disodium hydrogen phosphate
NaCl	sodium chloride
$NaHCO_3$	sodium bicarbonate
NaN_3	sodium azide
NaOH	sodium hydroxide
NBT	nitrotetrazolium blue chloride
NCBI	National Center for Biotechnology Information
ng	nanogramm
nm	nanometer
ns-LTP1	non-specific lipid transfer protein 1
OIV	The International Organisation of Vine and Wine
PAGE	polyacrylamide gel electrophoresis
PAU5	seripauperin 5
PBS	phosphate-buffered saline
pH	potential of hydrogen

pNPP	para-Nitrophenylphosphat
PR-proteins	pathogenesis-related proteins
PVDF	polyvinylidenflouride
PVP	polyvinylpyrrolidone
PVPP	polyvinylpolypyrrolidone
RP-HPLC	reversed-phase high performance liquid chromatography
rpm	round per minute
s	second
S. cerevisiae	*Saccharomyces cerevisiae*
SA	sinapinic acid
SDS	sodium dodecyl sulfate
spp.	species
TCA	trichloroacetic acid
TEMED	tetramethylethylenediamine
TFA	trifluoroacetic acid
TMW	Technische Mirkrobiologie Weihenstephan
UV	ultraviolet
V	volt
V. vinifera	*Vitis vinifera*
v/v	volume by volume
VDS	Verband deutscher Sektkellereien (German sparkling wine association)
w/w	weight by weight
λ	wavelength of electromagnetic radiation
°C	degree Celsius

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1. INTRODUCTION

1.1. Sparkling wines

Sparkling wines are characterized by their effervescence making it fizzy. After pouring, small bubbles rise through the liquid forming foam ringing the glass accompanied by the faint crackling sound of the escaping gas. The bubbles should be fine and persistent and are one of the most important quality characteristics of sparkling wines.

Sparkling wines became more and more important in the global market, especially in the market segment of grape derived alcoholic beverages in the last decades. From 2004 to 2014 the worldwide production of sparkling wines increased by 40 %. Whereas the worldwide wine production displayed an increase from about 10 % in the same period (OIV, 2014). Surveys showed that 301.6 million liters of sparkling wine were consumed in Germany in 2016 (Statistisches-Bundesamt, 2017). The total turnover generated by the sparkling wine sales from January 2026 to August in Germany was 5.42 billion €. The annual turnover calculated from this data was 8.13 billion € (Markant-Magazin, 2017). According to the German sparkling wine association (VDS) up to 2 % of the annual sparkling wine production can be affected by a phenomenon called gushing (personal communication VDS, 12. September 2017). Gushing in sparkling wines leads to costumer's reclamation or affected bottles are even not fed into the distribution system resulting in an economical loss for the companies. A detailed description of the gushing phenomenon is provided in section 1.2.

The worldwide production of sparkling wine differs in many details. Most countries have legal definitions that describe the level of carbonation as well as the carbonation process for sparkling wines, but they may not be consistent. A secondary alcoholic fermentation or malolactic fermentation as well as an artificially carbonation are common methods used for sparkling wine production.

According to the International Code of Oenological Practices (OIV) recommendations a sparkling wine is defined by an excess pressure of CO_2 of at least 3.5 bars at 20 °C. In addition, the effervescence is described to be a result of a secondary alcoholic fermentation in the bottle or in a closed tank (OIV, 2017). This thesis is mainly focusing on this type of sparkling wine. A brief description of sparkling wine production based on a secondary alcoholic fermentation is provided in the following section.

1.1.1. Sparkling wine production

The classification of sparkling wines depends on its production method. Variables like grape variety, sugar source or the vessel used for secondary fermentation, to name just a few, are deciding factors depending on complex legal regulations. The most common methods in sparkling wine production are categorized by bulk fermentation (Charmat method), bottle fermentation with filtration (transfer method) and the classic champagne method. A brief overview on those three production methods is given in Figure 1 and will be explained in the following.

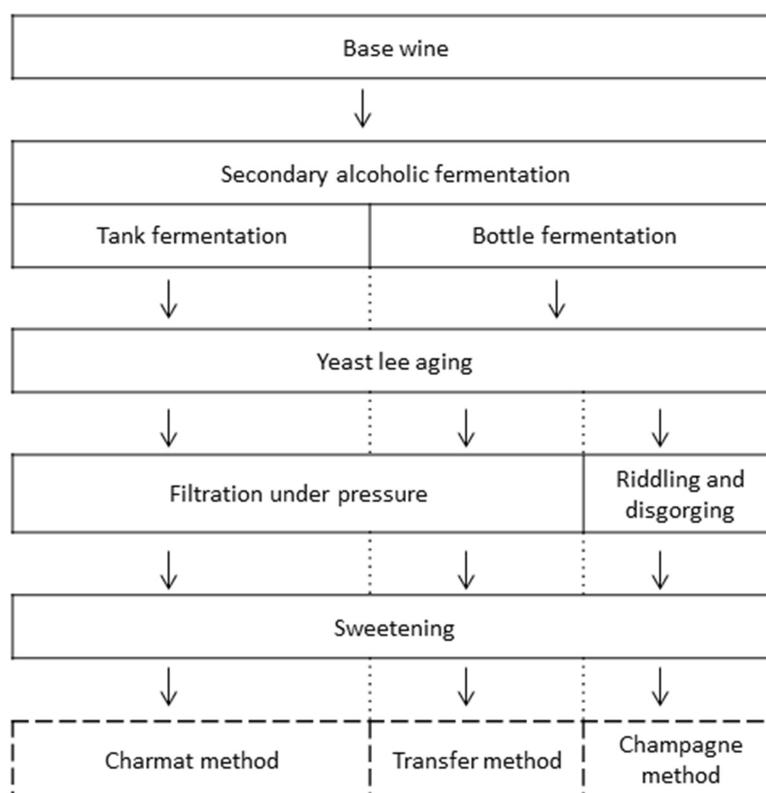


Figure 1: simplified schematic presentation of sparkling wine production

In all three production methods a base wine is used as a starting material where yeast is added converting sugar to CO_2. A brief description of the base wine making procedure is provided in section 1.1.2. In contrary to the first alcoholic fermentation carried out in base wine production, the secondary alcoholic fermentation takes place in a closed bottle or tank. The CO_2 produced by the yeast is trapped into the liquid resulting in carbonation. The sugar needed for secondary fermentation can be either residual sugar from the first fermentation or subsequently added from natural sugar sources like cane, beet, corn or grapes. If the fermentation is carried out in a tank the production procedure refers to the Charmat method.

Sparkling wines produced according to the transfer or Champagne method are fermented in sealed bottles. The fermentation temperature depends on its individual type of sparkling wine production method. For most of the champagne yeast the livable temperature ranges from 10 – 20 °C. After finishing the secondary fermentation, the sparkling wines are left to age in the closed tank or bottle on the yeast lees. This aging process induces several changes in the composition of sparkling wine compounds. Yeast autolysis and aging processes influence the quality and quantity of macromolecules like peptides, amino acids, lipids and polysaccharides which have an important effect on the sensory properties of the finished product. For racking off the yeast cells two different clarification methods are commonly used. Filtration under counterpressure is used if the secondary fermentation was carried out in a tank. Here the filtration step is accompanied with the bottling process. Clarification of sparkling wines fermented in bottles can be either done in the same way or by riddling and disgorging. If the transfer method is applied, the liquid is transferred to another bottle within the filtration under counterpressure. The Champagne method includes the riddling process. This work intensive procedure composed of turning and tilting movements of the bottles, results in an accumulation of the sediment in the bottle neck. The removal of the sediment, known as disgorging, is carried out by freezing the neck of the bottle and a subsequent opening. The internal pressure forces the frozen sediment out of the bottle and the clear liquid remains inside. The final taste of the sparkling wine is adjusted by the addition of a sugar containing liquid known as “dosage”. The composition of this syrup is determined by national and international regulations and the production method applied. It commonly consists of about 65 % pure sugar in grape wine and contains sulfur or other preservatives (Howe, 2003).

1.1.2. Base wine production

Base wines are referred to the wines used for sparkling wine production before secondary fermentation. The production follows the common winemaking parameters. A brief overview on white and red wine production is depicted in Figure 2.

For white wine production the grapes are crushed and pressed after harvesting. To prevent oxidation sulfur or ascorbic acid is added either immediately after harvesting or after crushing and pressing. The adjustment of the pH of the free-run juice is essential for optimal fermentation results. Therefore, acidification or de-acidification procedures are carried out. Residual grape solids are removed by the addition of pectolytic enzymes, cold settling or filtration- and centrifugation procedures. Processing mould-infected fruits requires in many

cases an additional treatment with fining agents like bentonite, PVPP or sodium caseinate after pressing the grapes. For the alcoholic fermentation *Saccharomyces (S.)* strains like *S. cerevisiae, S. uvarum* or *S. bayanus* are used. To enhance to fresh fruity wine style of white wines the fermentation temperature is maintained from 10 - 15 °C. It was shown that the presence of bentonite during fermentation in solid free juice results in higher fermentation rates, higher protein stability and ensures a rapid clarification after the fermentation. When the wine is racked off typical post-fermentation treatments are the addition of ascorbic acid to preserve the fruity character and treatment of sulfur for microbiological stabilization. For protein and tartrate stabilization a number of treatments can be applied. To remove heat-unstable proteins that are prone to cause protein haze, fining agents like bentonite are used. Tartrate stabilization can be carried out by cold stabilization with a subsequent filtration to remove precipitated particles. Hence white wines are sensitive to oxidation reactions; the oxidative stability can be improved by treating the wines with PVPP. Before bottling, commonly a final filtration step is carried out (Ewart, 2003).

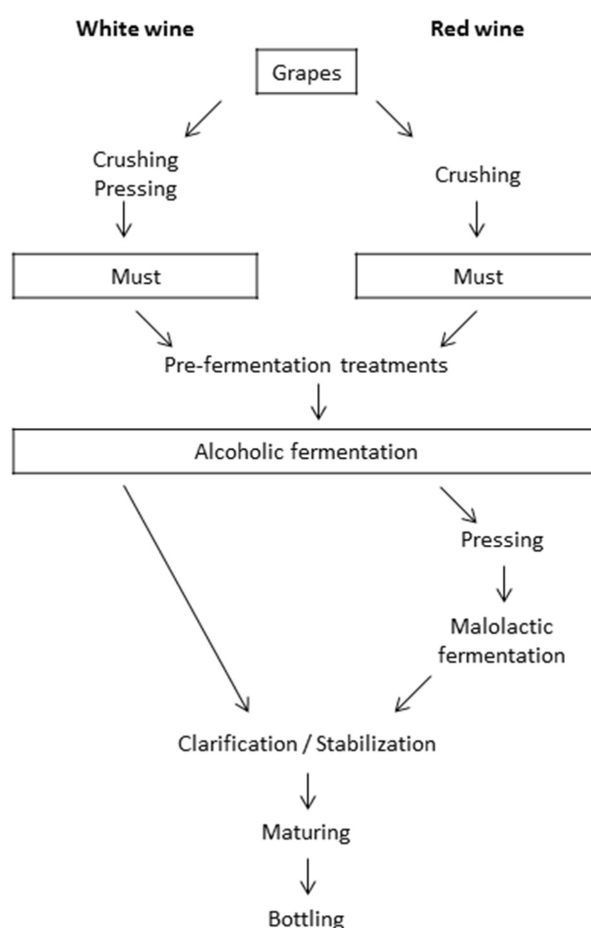


Figure 2: simplified schematic presentation of white and red wine production

Red wine differs mainly from the white wine by its extended contact to grape skin and seeds resulting in high amounts of phenolic compounds in the finished product. The must for fermentation consists of the crushed berries and is usually treated with sulfur to prevent spontaneous fermentation with natural occurring yeasts or oxidative processes induced by mould-infected material. In some cases, the must is left for 1 - 2 days in the temperature range from 15 - 20 °C. This operation is called maceration before fermentation that is enabling an extraction of seed and skin compounds without the presence of ethanol. The conventional maceration takes place during the fermentation process. Prior to yeast addition, the pH of the must is adjusted by addition of tartaric acid or carbonate salts and it is treated with additives like sulfur or pectolytic enzymes. After inoculation with the yeast the fermentation is carried out in the temperature range from 25 - 30 °C. When the fermentation is completed, an additional maceration can be carried out to change the mouth feel of the young wine. Here the fermentation tanks are closed and left standing for up to 3 weeks. To finish the maceration procedure the solid particles from grape skin and seeds are removed by pressing procedures and recovering of the free-run fraction. After the completion of the alcoholic fermentation the wines are transferred to barrels or tanks where the malolactic fermentation is initiated. During the malolactic fermentation malic acid is converted to lactic acid using *Leuconstoc*, *Lactobacillus* or *Pediococcus* strains. In the following aging process, the young wine undergoes several chemical modifications like the polymerization of anthocyanins or the enrichment of wood flavors from the barrels used. Various parameters like type of barrel or vessel, temperature or aeration can influence the aging process of the wine and consequently its sensory properties. In general, the red wines are filtrated before bottling to remove precipitates occurred during aging or residual microorganisms (Boulton, 2003).

1.2. Definition of gushing

Carbonated beverages can be affected by an unwanted phenomenon called gushing. It is defined as a spontaneous excessive over-foaming after pressure release which is generated by opening of a bottle or a can of beverage despite correct handling (Bach, 2001; Gjertsen, 1967; Kastner, 1909; Schumacher, 2002). Although it has no effect on the sensory properties of the beverage, it is perceived as a product failure by consumers. Therefore, the industry strives to avoid the occurrence of gushing to prevent economical losses and loss of company image. Many research groups dealing with gushing of beer have stated that this phenomenon must be caused by a complex interaction of gushing inducing and gushing preventing substances

(Garbe et al., 2009; Ilberg et al., 2009; Kieninger, 1983; Kunert et al., 2001; Winkelmann, 2004; Zapf et al., 2005).

1.3. Physical background of gushing

Carbonated beverages contain high levels of CO_2 present as saturated aqueous solutions. Sparkling wine contains up to 12 g/l CO_2. Whereas the CO_2 content of beer is ranging between 4-8 g/l depending on the type of beer. Fruit juice mixed with water (spritzers) is usually carbonated in the range from 4-6 g/l. In bottled beer, most of the CO_2 (98 % - 99 %) is dissolved in the liquid and only a small amount is present in its undissociated form (H_2CO_3) and as bicarbonates since CO_2 solubility in aqueous environment depends on the pH of the aqueous solution (Ferdinandus et al., 1962; Kunert et al., 2001; Liger-Belair, 2006; Schulze, 1985; Schumacher, 2002). CO_2 in its gaseous form is known to be the driving force in the development of gushing. A slow bubble release without over-foaming can be observed by opening a bottle of carbonated beverage. If the product is affected by gushing, the CO_2 release is increased resulting in an over-foaming of the liquid. Beside factors such as temperature or the density of the liquid, other influences leading to an excessive bubble growth and formation are discussed as a reason for the development of gushing and will be explained in the following chapter (Gjertsen et al., 1963; Pellaud, 2002; Vaag et al., 1993)

1.3.1. Bubble formation

According to Fischer (2001), the formation of gushing relevant gas bubbles can be divided into the formation from "de novo nuclei" or the formation from pre-existing gas nuclei as depicted in Figure 3. The bubble formation with "de novo nuclei" is divided into homogenous and heterogeneous bubble formation. Bubble formation in an oversaturated liquid without nuclei such as particles or already existing gas bubbles is defined as homogenous "de novo nuclei" bubble formation which appears depending on pressure and temperature. However, this mechanism plays a minor role as a factor in gushing induction since the conditions necessary for the development of a spontaneous bubble formation, about 100 times over saturated liquid, are usually not present in carbonated beverages. As an example, over-carbonation of beer can in rare cases lead to homogenous "de novo nuclei" bubble formation resulting in unwanted over-foaming. However, in this case the beer is not classified as gushing beer. Solid particles acting as nuclei are rated as heterogeneous "de novo" bubble formation. Particles in the liquid or irregularities of the inner glass surface of the bottle can act as nuclei of that type. Gas cavities that may occur on irregularities of the

inner bottles surface or microbubbles already existing in the carbonated liquid, e.g. from the filling process, are also potential nuclei for bubble growth. Particles, irregularities of the bottle, microbubbles or gas cavities do not act as nuclei for bubble formation in every case. Indeed, the geometry of particles or the contact angle play an important role in nuclei induced bubble formation (Aquilano et al., 2003; Christian et al., 2009; Fischer et al., 1997; Jones et al., 1999; Liger-Belair, 2003; Lubetkin, 1994; Pahl & Ozkurt, 1998)

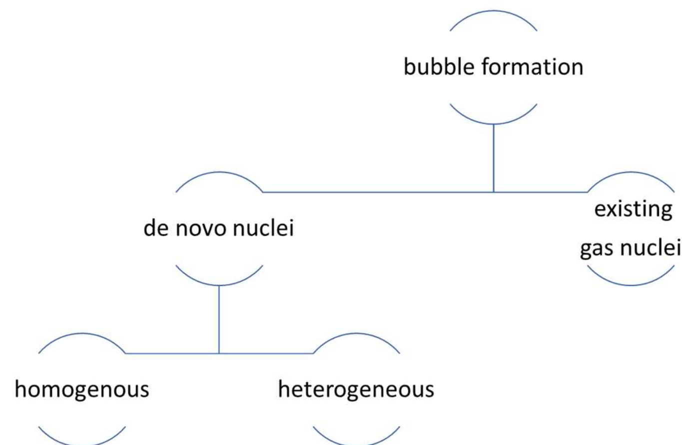


Figure 3: Mechanisms of bubble formation according to Fischer (2001)

1.3.2. **Stabilization of microbubbles**

As mentioned in the previous section, microbubbles can act as nuclei for bubble formation and growth and are discussed in the literature as a possible reason for the development of gushing in beer. Movements of the liquids as it happens during the bottling or transport process may lead to the development of microbubbles (Thorne & Helm, 1957). Surface-active substances can adsorb at the air/water interface of such microbubbles and lower the surface tension as well as the gas permeability (Pellaud, 2002). This layer of surface-active substances acts like a stabilizing membrane around the bubble. Those bubbles remain in the liquid for a long time if they are small enough not to rise to the surface due to their low buoyancy (Yount et al., 1984). Those microbubbles stabilized by surface-active molecules are well known nuclei for bubble formation in gushing beverages (Christian et al., 2011).

1.4. Causes for gushing on the example of beer

So far most of the research has been done on gushing in beer. This research showed that gushing can be caused by complex reactions of different factors as reviewed by Shokribousjein et al. (2011). The most common differentiation of causes for beer gushing is to divide between primary gushing which is induced by substances originating from the raw material and secondary gushing which is caused by factors prevailing during the production process (Gjertsen, 1967; Gjertsen et al., 1963).

1.4.1. Primary gushing

Many studies regarding primary gushing in beer revealed the importance of surface-active proteins in the induction of this phenomenon. Proteins influencing gushing as stabilizers or as inducers were identified as proteins produced by the barley plant like the non-specific lipid transfer protein 1 (ns-LTP1) and fungal proteins called hydrophobins that are introduced into the beverage during the mashing process (Ilberg et al., 2009; Laitila et al., 2007; Niessen et al., 2006)

An infection of the malt with filamentous fungi such as *Fusarium* spp. is highly associated with the occurrence of primary gushing (Gjertsen et al., 1965; Gyllang & Martinson, 1976; Haikara, 1983; Schwarz et al., 1996; Sloey & Prentice, 1962). Those filamentous fungi produce surface-active proteins such as hydrophobins, which were identified as gushing-inducing proteins. Hydrophobins are small cysteine rich proteins with an amphiphilic character (Amaha et al., 1973; Chandler, 2005; Kleemola et al., 2001; Linder et al., 2005; Lutterschmid et al., 2010; Sarlin et al., 2005). Due to their amphiphilic nature, they can stabilize air microbubbles in liquids as described in section 1.3.2 by self-assembling at air/water interfaces (Cox et al., 2007; De Vocht et al., 1998; Gruner et al., 2012; Linder, 2009). Kitabatake et al. (1980) identified another gushing inducing molecule a cyclic tetrapeptide from *Penicillium chrysogenum*. Moreover, *Nigrospora* spp., *Stemphylium* spp. and *Rhizopus* spp. are assumed to produce gushing inducing polypeptides (Amaha et al., 1973; Kitabatake & Amaha, 1977).

Plant proteins such as the non-specific lipid transfer protein 1 (ns-LTP1) and the protein Z from *Hordeum (H.) vulgare* are known to influence the foaming properties of beer. Protein Z is responsible for beer foam stability, whereas ns-LTP1 has more influence on foam formation. Ns-LTP1 from *H. vulgare* consists of 91 amino acids with a basic isoelectric point from pH 8-10 depending on its glycations (García-Casado et al., 2001; Leiper et al., 2003;

Sorensen et al., 1993; Stanislava, 2007). In its native state, ns-LTP1 displays poor foaming characteristics. During the brewing process it undergoes chemical modifications such as glycation resulting in an increased amphiphilic character and increased foaming properties (Jégou et al., 2000).

Hippeli and Hecht (2009) detected reduced amounts of ns-LTP1 in gushing beer compared to non-gushing beer of comparable negative controls and postulated a degradation of this protein by fungal proteases during the brewing process as a cause for beer gushing. The addition of transgenic hydrophobin from *Fusarium culmorum* (FcHyd5p) in a constant concentration to a non-gushing beer resulted in high gushing volumes which were reduced significantly in a dose-dependent manner when transgenic glycated ns-LTP1 was added (Lutterschmid et al., 2011). In the study of Specker et al. (2014) similar observations were made by the addition of protein Z to gushing beer.

From the results, the authors deduced that the level of gushing might rather depend on the proportion of gushing-inducing (hydrophobin) and gushing-stabilizing (ns-LTP1) proteins than on the concentration of the gushing inducer alone.

Lipid transfer proteins are categorized into the class of pathogenesis-related proteins (PR-proteins) and are ubiquitous plant proteins. The ns-LTP1 proteins share a high sequence homology and conserved structures in all plants (Kader, 1996; Van Loon & Van Strien, 1999). All of them have 8 cysteine residues and a secondary structure of 4 α-Helices stabilized by four intramolecular disulfide bridges (Guerbette et al., 1999; Salcedo et al., 2004).

Beside fungal and plant proteins also hop compounds are discussed in the literature to be gushing influencing substances. Dehydrated humulinic acid, oxidation products of iso α-acids and reduced isohumulones are described so far as potentially gushing inducing molecules (Carrington et al., 1972; Laws & McGuinness, 1972). As this thesis focus on surface-active proteins that influence the gushing potential of sparkling wine, non-proteinic substances will not be described here in more detail.

1.4.2. **Secondary gushing**

Particles acting as nuclei for bubble formation were already identified in beer. Calcium reacting with oxalic acid results in the occurrence of calcium oxalate crystals that are known to be a possible nucleation site and therefore a gushing inducing factor (Zepf & Geiger,

2000). Ferdinandus et al. (1962) as well as Weideneder (1992) reported particles acting as nuclei for bubble formation originating from the crown caps. Other small particles causing bubble formation in beer are cleaning agent residuals, metal ions or residual filter aids (Dachs & Nitschke, 1977; Rudin & Hudson, 1958; Zarnkow & Back, 2001). As already mentioned in section 1.3.1, a rough surface of the inner bottle wall is also a potential cause for the development of gushing (Rammert & Pahl, 1992). The formation of protein haze or even not visibly detectable protein coagulates are also discussed to be gushing inducing nuclei of the secondary type (Bach, 2001; Curtis & Martindale, 1961)

1.5. Causes for gushing in sparkling wine

The influence of proteins on the development of gushing in sparkling wine has not been investigated to any similar extent as in beer. So far, only one study has been published dealing with the influence of proteins on the development of gushing in sparkling wine. According to Bach et al. (2001), the addition of mannoproteins to sparkling wine may lower the tendency for gushing. However, in this study no detailed characterization of those proteins regarding their gushing inhibiting properties was carried out.

Particles in sparkling wine were also assumed to be responsible for the unwanted over-foaming after pressure release. According to the research of Schanderl (1964), cork and fungal spores can provoke gushing in sparkling wine. In addition, metal ions, tannins, filter aids and crystals were found to be inducers of secondary gushing (Hennig, 1963; Kielhöfer & Würdig, 1961; Liger-Belair et al., 2013; Würdig & Müller, 1979). As briefly reviewed by Vogt et al. (2017)

1.6. Proteins in wine and sparkling wine

1.6.1. Current methods of analysis and determination of the concentration

An exact determination of the protein concentration proves to be difficult as proteins in must, wine and sparkling wine are present in extremely small quantities. The low protein levels in wine and sparkling wine are caused by the proteolysis and denaturation that occur during the fermentation process (Murphey et al., 1989; Waters et al., 1992). Several research groups already calculated the protein concentration with methods like the Bradford assay (Bradford, 1976). This method is a tool commonly used in laboratories. However, in the case of must, wine and sparkling wine the response of the different proteins to the Bradford reagent is extremely different and no standard wine proteins are available for the generation of a

standard curve. Therefore protein concentrations are usually expressed as BSA equivalents per liter of wine. Esteruelas et al. (2009) found up to 100 BSA equivalents per liter in a Sauvignon white wine. Somers and Ziemelis (1973) calculated the protein concentration in white wine by chromatography using a Sephadex G-25 column with a determination of the peak areas obtained at 280 nm and found concentrations up to 840 mg/l but the interfering phenolic compounds bound to the wine proteins can result in an overestimation. The described difficulties in the determination of the protein concentration increase the errors in both measurement methods. Consequently, the values obtained should be considered as an indication for the protein concentration rather than absolute quantities.

Due to the low concentration of proteins and high amount of interfering substances, the protein analysis in must, wine and sparkling wine is challenging and the methods applied should be chosen with care. So far, several methods using FPLC, HPLC, size exclusion chromatography, affinity chromatography, SDS-PAGE, two-dimensional electrophoresis or immunoblotting were applied for the characterization of the wine and sparkling wine proteins (D'Amato et al., 2010; Dawes et al., 1994; Dizy & Bisson, 1999; Dorrestein et al., 1995; Ferreira et al., 2000; Hsu & Heatherbell, 1987b; Lamikanra & Inyang, 1988; Ledoux et al., 1992; Monteiro et al., 1999; Pueyo et al., 1993; Santoro, 1995; Tyson et al., 1981; Waters et al., 1993; Waters et al., 1992; Wigand et al., 2009).

Most of the analyses conducted in wine and sparkling wine were carried out with white (sparkling) wines. This can be contributed to the fact that red (sparkling) wines contain even higher amounts of interfering phenolic substances which makes protein analysis even more challenging.

1.6.2. Origin of (sparkling) wine proteins

Proteins in the size range of 9 to 80 kDa with isoelectric points ranging from 3-9 were found in wines and sparkling wines (Brissonnet & Maujean, 1993; Cilindre et al., 2014; Hsu & Heatherbell, 1987b; Lamikanra & Inyang, 1988). Most of the soluble wine proteins were found to be low molecular weight proteins (20-30 kDa) with low isoelectric points ranging from 4.1 to 5.8 (Brissonnet & Maujean, 1993; Ferreira et al., 2000). Some previous studies concluded that the majority of wine proteins are glycosylated (Paetzold et al., 1990; Yokotsuka et al., 1994). However, no detailed characterization of glycosylated proteins in wine and sparkling wine was carried out.

Kwon (2004) identified proteins in a Sauvignon Blanc wine originating from the grape (*Vitis (V.) vinifera*), the yeast, or from bacteria and fungi. In other studies, the proteins were found to originate from the grape, the yeast and the grape associated fungus *Botrytis (B.) cinerea* (Cilindre et al., 2014; Cilindre et al., 2008; Okuda et al., 2006). According to the research of Ferreira et al. (2000) most of the wine proteins originate from the grape pulp. Many research groups stated that also the grape variety, "Terroir" and biotic stress have an impact on the protein composition and concentration in the resulting wine (Bayly & Berg, 1967; Dizy & Bisson, 1999; Dorrestein et al., 1995; Monteiro et al., 2003; Pueyo et al., 1993; Wigand et al., 2009). PR-proteins, like chitinases, thaumatin-like proteins and osmotin were mainly identified as grape derived proteins in wines (Esteruelas et al., 2009; Le Bourse et al., 2011; Marangon et al., 2009; Peng et al., 1997; Waters et al., 1996).

Proteins from the yeast (*S. cerevisiae*) are less abundant in (sparkling) wine than the grape derived proteins (Cilindre et al., 2014; Cilindre et al., 2008; Dambrouck et al., 2003). Yeast proteins get into the beverage either by active secretion or during the autolysis process of the yeast cells. The yeast derived protein composition depends on the choice of the strain as well as on the secondary fermentation and the aging process (Martinez-Rodriguez et al., 2002; Martinez-Rodriguez et al., 2001).

1.6.3. **Proteins influencing foaming characteristics in sparkling wine**

Although the role of proteins on gushing in sparkling wine has hardly been investigated, their importance regarding foam properties of the beverage has been studied in more detail. Several research groups showed a positive correlation between protein concentration and the foaming properties of (sparkling) wine (Malvy et al., 1994; Pueyo et al., 1995). Brissonnet and Maujean (1993) found that proteins contributing to foam-formation of a Champagne base wine were hydrophobic. Glycoproteins are described as the predominant protein fraction in the foam of wine (Dambrouck et al., 2003). A positive correlation was found between the content of invertase as a grape-derived glycoprotein, and foam quality (Dambrouck et al., 2005). So far, yeasts are known to be the major source for foam-active glycoproteins, i.e. mannoproteins in sparkling wine (Blasco et al., 2011; Núñez et al., 2006; Núñez et al., 2005). Mannoproteins are introduced into the beverage during the autolysis process of the yeast cells. It was stated that yeast derived mannoproteins have an amphiphilic character resulting in foam stabilizing properties due to their hydrophilic sugar chains and hydrophobic part of amino acids, although there was no detailed characterization of foam-stabilizing

mannoproteins carried out so far (Blasco et al., 2011; Gonzalez et al., 2003; Marchal et al., 1996; Senée et al., 1999). The importance of the yeast on the foaming properties of sparkling wine was also confirmed by other research groups. Núñez et al. (2005) and Martıinez-Rodrıiguez et al. (2001) found that the yeast strain used for the second fermentation and an accelerated autolysis of the yeast alters the foam quality. Nevertheless, no detailed examination on the foam influencing proteins or molecules was carried out in those studies. Beside the positive influence on the foam quality some research groups stated a protective effect of mannoproteins against tartaric crystals and haze formation (Gerbaud et al., 1997; Lubbers et al., 1993; Moine-Ledoux et al., 1997).

1.7. The influence of a *B. cinerea* infection on protein composition and foam quality

According to the findings of Marchal et al. (2001) an infection of the grapes with *B. cinerea* leads to dramatic changes in the foaming properties of base wines used for Champagne production. Cilindre et al. (2007) reported that an infection of grapes with *B. cinerea* resulted in altered foaming properties as well as in altered protein composition in a Champagne base wine. Analysis of the botrytized wine by SDS-PAGE and immunoblotting revealed that besides the occurrence of new proteins, some plant proteins were less abundant compared to the negative control. The reduced amount or even absence of some plant proteins, especially those with a molecular weight lower than 23 kDa, in a botrytized Champagne base wine was verified in a follow up study (Cilindre et al., 2008). It was assumed that protein degradation is related to the proteolytic activity of *B. cinerea* (Ten Have et al., 2004). A decrease in foamability accompanied by a degradation of plant proteins caused by the proteolytic activity of *B. cinerea* was also confirmed in synthetic wine (Marchal et al., 2006).

1.8. The influence of bentonite fining on protein composition and foam quality

Bentonite is the most common clarifying agent in wine production. It helps to eliminate off-flavors as well as the wine's ability to oxidize and it removes proteins that play a role in haze formation in wines. Bentonite can be added at different steps in the production process. This fining agent is acting as a cation exchanger where the positively charged wine proteins interact electrostatically with the negative charge of the bentonite. The bound components are removed together with the bentonite from the wine (Hsu & Heatherbell, 1987a; Lamikanra &

Inyang, 1988). According to Dambrouck et al. (2005), the bentonite treatment of wine results in a significant decrease of total protein accompanied by a loss of foaming properties. In contrast, the treatment with casein has less influence on the protein content and foamability. The authors stated the importance of wine proteins in foam formation since they found a correlation between the decrease of total protein with the decrease of the foaming properties. These results were confirmed by Marchal et al. (2002) who found a diminished foamability of Champagne base wines after bentonite treatment. Vanrell et al. (2007) stated a decrease of protein and a reduction of foamability by using bentonite as a riddling agent on sparkling wine production. Protein removal by the use of bentonite was also observed by Sauvage et al. (2010). However, no examinations regarding the foaming properties were carried out in their study.

1.9. Motivation and objectives of this work

Spontaneous over-foaming of carbonated beverages after pressure release upon opening of the bottle is known as gushing. This unwanted phenomenon results in adverse financial consequences and an image loss for the affected producers. In Germany up to 2% percent of the annual sparkling wine production can be affected by this phenomenon. The sparkling wine producers strive to avoid the occurrence of gushing to prevent economic loss. Therefore, part of this work was supported by the German Ministry of Economics and Technology via the German Federation of Industrial Research Association and the German Sparkling wine association. To prevent the occurrence of gushing, the elicitors need to be identified. Many previous studies emphasized the importance of surface-active proteins for the development of gushing in beer. However, the cause for the development of this phenomenon in sparkling wine has hardly been investigated. Previous research has shown that a *B. cinerea* infection of the grapes as well as finning with bentonite influences the protein composition of wine and its foaming properties. The exact identity and the contribution of proteins to the foaming properties of sparkling wine and to the presence or absence of gushing have not yet been fully elucidated.

Hypothesis:

The gushing phenomenon in sparkling wine is caused by similar or identical elicitors than those found in beer. Surface-active proteins in sparkling wine originating from the raw material or the yeast have an impact on the foam quality and act in analogy to the gushing preventing or inducing proteins found in beer.

This hypothesis leads to the following objectives:

- Identification of particles that may cause secondary gushing
- Development and optimization of protein purification and analysis strategies in wine and sparkling wine
- Protein chemical comparison of a gushing and non-gushing sparkling wine
- Identification of proteins that correlate with the absence or presence of gushing with a further characterization of those proteins regarding to their foaming properties
- Identification of proteins in sparkling wine that are known to be gushing influencing from the gushing research done in beer
- Investigation of the influence of process parameters on the concentration of those proteins

2. MATERIAL AND METHODS

2.1. Materials

2.1.1. Chemicals

Chemicals used for the current work are listed in Table 1.

Table 1: list of chemicals used in the current work

chemical	purity grade	manufacturer
2-Butanol, ≥ 99 %	≥ 98.5 %, for synthesis	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
2-Mercaptoethanol	BioReagent, 99 %	Sigma-Aldrich GmbH, Schnelldorf, Germany
2-Propanol	≥ 99.5 %, for synthesis	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
5-Bromo-4-chloro-3-indolyl phosphate (BCIP) toluidine salt		Carl Roth GmbH + Co. KG, Karlsruhe, Germany
Acetic acid	Rotipuran®, 100 %, p.a.	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
Acetone	≥ 99 %	FLUKA, Sigma-Aldrich GmbH, Steinheim, Germany
Acetonitrile (ACN)	Rotisolv®, HPLC gradient grade	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
Acrylamide /bis solution 29:1	(30 % w/v), 3.3 % C	SERVA Electrophoresis GmbH, Heidelberg Germany
Agar-Agar	bioscience-grade, powdered	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
Agarose Biozym LE	for electrophoresis	Biozym Scientific GmbH, Hessisch Oldendorf Germany
Albumin fraction V (bovine serum albumin (BSA))	≥ 98 %	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
Ammonium acetate	≥ 96 %, research grade	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
Ammonium hydrogen carbonate	purum, p.a., ≥ 99 %	FLUKA, Sigma-Aldrich GmbH, Steinheim, Germany
Ammonium persulphate (APS)	analytical grade	SERVA Electrophoresis GmbH, Heidelberg Germany
Ammonium sulphate	high purity	GERBU Biotechnik GmbH, Heidelberg, Germany
Bentonite	pure	Carl Roth GmbH + Co. KG, Karlsruhe, Germany

chemical	purity grade	manufacturer
Berol 532		Julius Hoesch GmbH & Co. KG, Düren, Germany
Berol 840		Akzo Nobel Surface Chemistry AB, Stenungsund, Schweden
Bromphenol blue	for electrophoresis	AppliChem GmbH, Darmstadt, Germany
Diethanolamine		Merck KGaA, Darmstadt, Germany
Dimethylformamide (DMF)	≥ 99 %	Sigma-Aldrich GmbH, Schnelldorf, Germany
Dipotassium hydrogen phosphate trihydrate (K_2HPO_4 * $3H_2O$)	for analysis, Emsure®	Merck KGaA, Darmstadt, Germany
Disodium hydrogen phosphate dehydrate (Na_2HPO_4 * $2H_2O$)	≥ 98 %, Ph. Eur., USP	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
Dithiothreitol (DTT)	high purity	GERBU Biotechnik GmbH, Heidelberg, Germany
Ethanol	≥ 94 %, completely denaturated	CLN GmbH, Niederhummel, Germany
Ethanol absolute	ACS Rea., Ph. Eur.	VWR International GmbH, Darmstadt Germany
Ethylendiaminetetra-acetic acid (EDTA) disodiumsalt dihydrate		GERBU Biotechnik GmbH, Heidelberg, Germany
FireSilver staining kit	compatible with mass spectrometry	Proteome Factory AG, Berlin, Germany
Formaldehyde (37 %)	≥ 37 %, for synthesis	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
Glucose monohydrate	for microbiology	Merck KGaA, Darmstadt, Germany
Glycerol (87 %)	high purity, Ph. Eur.	GERBU Biotechnik GmbH, Heidelberg, Germany
Glycerol (99 %)	high purity	GERBU Biotechnik GmbH, Heidelberg, Germany
Glycine	for mol. biology & electrophoresis	GERBU Biotechnik GmbH, Heidelberg, Germany
Hydrogen chloride (HCl)		Carl Roth GmbH + Co. KG, Karlsruhe, Germany
Hydrochloric acid, fuming (37 %)	37 %, techn.	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
Magnesium chloride ($MgCl_2$)	≥ 98.5 %, anhydrous	Carl Roth GmbH + Co. KG, Karlsruhe, Germany

chemical	purity grade	manufacturer
Magnesium sulphate heptahydrate	p.a. ACS, Reag. Ph. Eur., Emsure®	Merck KGaA, Darmstadt, Germany
Malt extract		AppliChem GmbH, Darmstadt, Germany
Methanol	Rotisolv®, HPLC gradient grade	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
Nitrotetrazolium blue chloride (NBT)	≥ 98 %, p.a.	Sigma-Aldrich GmbH, Schnelldorf, Germany
Organic solvent (ACN 50 %, water 47.5 %, TCA 2.5 %)	for mass spectrometry	Honeywell Speciality Chemicals Seelze GmbH, Seelze, Germany
Ortho phosphoric acid (85 %)	Rotipuran®, p.a., ACS, ISO	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
Para-Nitropenylphosphat (pNPP)		Thermo Scientific Inc., St. Leon-Rot, Germany
Perchloric acid	70 %	Sigma-Aldrich GmbH, Schnelldorf, Germany
Polyethylene glycol 20000	Rotipuran®, Ph. Eur.	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
Polyethylene glycol 8000		Sigma-Aldrich GmbH, Schnelldorf, Germany
Polyvinylpolypyrrolidone (PVPP)	~110 µm particle size	FLUKA, Sigma-Aldrich GmbH, Steinheim, Germany
Polyvinylpyrrolidone (PVP) 360		Sigma-Aldrich GmbH, Schnelldorf, Germany
Potassium chloride (KCl)	≥ 99 %, Cellpure®	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
Potassium dihydrogen orthophosphate	for analysis, Emsure®, ISO	Merck KGaA, Darmstadt, Germany
Potassium metabisulfite		Kadifit, Erbslöh, Geisenheim Germany
Proteinase K	2 units/mg protein Recombinant PCR-Grade	Roche, Penzberg, Germany
Roti®-aqua-phenol	Water saturated phenol	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
Silver nitrate	≥ 99.9 %, p.a.	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
Sinapinic acid (SA)		Bruker Daltonics GmbH, Bremen, Germany
Sodium azide (NaN_3)	for synthesis	Merck KGaA, Darmstadt, Germany

chemical	purity grade	manufacturer
Sodium bicarbonate ($NaHCO_3$)	≥ 99 %, Cellpure®	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
Sodium carbonate (Na_2CO_3)	≥ 99.9 %, p.a., ACS	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
Sodium chloride (NaCl)	≥ 99.9 %, p.a., ACS	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
Sodium dodecyl sulfate (SDS) in pellets	research grade	SERVA Electrophoresis GmbH, Heidelberg Germany
Sodium hydroxide	≥ 99 %, p.a., ISO	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
Sodium thiosulfate pentahydrate	p.a., ACS, ISO	Merck KGaA, Darmstadt, Germany
Soy peptone (PEPTONE EX SOYA)	papainic digest, for microbiology	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
Spectra™ multicolor low range protein ladder		Thermo Fisher Scientific Inc., Waltham, USA
Sucrose		Carl Roth GmbH + Co. KG, Karlsruhe, Germany
Tetramethylethylenediamine (TEMED)	~99 %	Sigma-Aldrich GmbH, Schnelldorf, Germany
Thio urea	p.a., ACS, Reag. Ph. Eur.	Merck KGaA, Darmstadt, Germany
Trichloroacetic acid (TCA)	p.a. ACS, Reag. Ph. Eur., Emsure®	Merck KGaA, Darmstadt, Germany
Tricine	Pufferan®, ≥ 99 %	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
Triflourmethanesulfonic acid	~98 %	Sigma-Aldrich GmbH, Schnelldorf, Germany
Trifluoroacetic acid (TFA)	Chromasolv®, ≥ 99 %, for HPLC	Sigma-Aldrich GmbH, Schnelldorf, Germany
Tris-HCl	for mol. biology	GERBU Biotechnik GmbH, Heidelberg, Germany
Tris-X	ultra pure	GERBU Biotechnik GmbH, Heidelberg, Germany
Triton®X-100	for mol. biology	Sigma-Aldrich GmbH, Schnelldorf, Germany
Trypton/pepton from casein	Pancreatic digest	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
Tween 20	cell culture and bacteriology grade	GERBU Biotechnik GmbH, Heidelberg, Germany

chemical	purity grade	manufacturer
Urea	ultra pure	GERBU Biotechnik GmbH, Heidelberg, Germany
Water	HPLC gradient grade	J.T. Baker, Center Valley, USA
Yeast extract		Carl Roth GmbH + Co. KG, Karlsruhe, Germany
α-Cyano-4-hydroxycinnamic acid (HCCA)		Bruker Daltonics GmbH, Bremen, Germany

2.1.2. **Equipment**

Equipment used for the current work are listed in Table 2

Table 2: list of equipment used in the current work

equipment	type	manufacturer
1D-gel electrophoresis	Mini-PROTEAN® Tetra Cell PowerPac™ Basic Power Supply	Bio-Rad Laboratories GmbH, Munich, Germany
Analytical balance	SI-234	Denver Instrument, Sartorius, Bohemia, NY USA
Centrifuge	Sigma 1 K15	Sigma Labortechnik, Osterrode am Harz, Germany
Centrifuge	Lab centrifuge J-6	Beckman, Paolo Alto, CA, USA
Centrifuge	Microzentifuge	Hermle Labortechnik GmbH, Wehingen, Germany
Freeze dryer	FreeZone2.5plus	Labcono, Kansas City, USA
Gel-scanner	Bio-Microtec 5000	Serva Electrophoresis GmbH, Heidelberg, Germany
Hemocytometer	Thoma type, 0.1 mm chamber depth	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
HPLC autosampler	UlitMate3000™ autosampler	Dionex/Thermo Fisher Inc., Germering, Germany
HPLC column	Aeris PEPTIDE 3.6u XB-C18 250 x 2.1 mm	Phenomenex, Aschaffenburg, Germany

equipment	type	manufacturer
HPLC column department	Thermostated Column Compartment TCC-100	Dionex/Thermo Fisher Inc., Germering, Germany
HPLC pump	UlitMate3000™	Dionex/Thermo Fisher Inc., Germering, Germany
HPLC wavelength detector	UlitMate3000™ variable wavelength detector	Dionex/Thermo Fisher Inc., Germering, Germany
MALDI-TOF MS	microflex LT	Bruker Daltonics GmbH, Bremen, Germany
MALDI-target	MSP 96 polished steel, microscout target	Bruker Daltonics GmbH, Bremen, Germany
Plate reader	Emax	Molecular Devices, Sunnyvale, CA USA
Semi-dry blotter	EP-1	peqLab, Erlangen, Germany
Round-bottom flask	50 ml	Schott AG, Mainz, Germany

2.1.3. Consumables

Consumables used for the current work are listed in Table 3

Table 3: list of consumables used in the current work

product	type	manufacturer
24 Well TC Platte	Standard F	Sarstedt, Nürnbrecht, Germany
96-well microtiter plates	Maxisorp	Thermo Scientific Inc., St. Leon-Rot, Germany
Blotting paper		Munktell, Billerica, MA, USA
Cannula	Sterican 0.60 x 30/80 mm	B. Braun Biotech International, Melsungen, D.
Centrifugation tubes	15 and 50 ml	Sarstedt, Nürnbrecht, Germany
Dialysis tubes	MWCO 3500 Da, Ø 16 mm	Serva, Heidelberg, Germany
HPLC vials	Vrex Vial 2 ml	Phenomenex, Aschaffenburg, Germany
Petridishes		Sarstedt, Nürnbrecht, Germany
PVDF membrane	Immuno-Blot™	Bio-Rad Laboratories GmbH, München, D.

product	type	manufacturer
Muslin bandage	100% polyester	Altapharma Naturprodukte GmbH, Hamburg, D.
Reaction tubes	200 µl, 1.5 µl and 2.0 ml	Eppendorf, Hamburg, Germany
Srynge filter	Nylon membrane	Phenomenex, Aschaffenburg, Germany
Steril filter	Filtropur S 0.45	Sarstedt, Nürnbrecht, Germany
Vial insert		TECHLAB, Braunschweig, Germany

2.2. Buffers, solutions and media

2.2.1. **Buffers**

2.2.1.1. Protein purification

Buffer 1	
0.1 M	Tris base
10 mM	EDTA
0.4 % (v/v)	2-mercaptoethanol
100 mM	KCl
10 % (w/v)	DTT (freshly prepared)
pH 8.9	adjusted by addition of HCL
Buffer 1_G	
0.1 M	Tris base
10 mM	EDTA
0.4 % (v/v)	2-mercaptoethanol
0.8 M	Sucrose
10 % (w/v)	DTT (freshly prepared)
pH 8.9	adjusted by addition of HCL
Buffer 2	
0.1 M	Ammonium acetate
dissolved in 100 %	Methanol
Buffer 3	
0.1 M	Ammonium acetate
10 mM	DTT
dissolved in 100 %	Methanol
Buffer 4	
10 mM	DTT
80 % (v/v)	Acetone

2.2.1.2. SDS-PAGE according to Schägger and Von Jagow (1987)

Separating gel (16 % acrylamide; 1.0 M Tris; pH 8.45)	
5.30 ml	Acrylamide /bis solution 29:1
3.33 ml	Gel buffer
1.26 ml	Deionized H_2O
40 µl	SDS-solution, 25 % (w/v)
7µl	TEMED
50 µl	APS, 10 % (w/v)
Stacking gel (4 % acrylamide; 0.74 M Tris; pH 8.45)	
0.68 ml	Acrylamide /bis solution 29:1
1.28 ml	Gel buffer
3.21 ml	Deionized H_2O
16 µl	SDS-solution, 25 % (w/v)
7µl	TEMED
33 µl	APS, 10 % (w/v)
Gel buffer (3.0 M Tris, pH 8.45)	
90.86 g	Tris-base, dissolved in 250 ml deionized H_2O
pH 8.45	adjusted by addition of HCL
5x-Cathode buffer (0.5 M Tris; 0.5 M Tricine; 0.5 % SDS, pH 8.25)	
60.57 g	Tris-base
89.58 g	Tricine
20 ml	SDS-solution, 25 % (w/v)
adjusted to 1000 ml with deionized H_2O	
5x-Anode buffer (1 M Tris; pH 8.9)	
121.14 g	Tris-base
pH 8.9	adjusted by addition of HCL
adjusted to 1000 ml with deionized H_2O	
2x-Application buffer	
250 mM	Tris, pH 8,45
7.5 % (w/v)	SDS
25 % (v/v)	Glycerol
0.25 mg/ml	Bromphenol blue
12.5 % (v/v)	2-mercaptoethanol

2.2.1.3. Solutions for silver staining of protein gels according to Blum et al. (1987)

Fixative	
40 ml	Ethanol
10 ml	Acetic acid
50 ml	deionized H_2O
Wash solution	
30 ml	Ethanol
70 ml	deionized H_2O
Thiosulfate reagent	
20 mg	Sodium thiosulfate
adjusted to 100 ml with deionized H_2O	
Silver nitrate reagent	
0.2 g	Silver nitrate
adjusted to 100 ml with deionized H_2O	
Developer	
3 g	Sodium carbonate
0.5 mg	Sodium thiosulfate
adjusted to 100 ml with deionized H_2O	
100 µl	Formaldehyde 37 %
	(add immediately prior to use)
Stop reagent	
0.5 g	Glycine
adjusted to 100 ml with deionized H_2O	

2.2.1.4. Buffers for reversed phase high pressure liquid chromatography (RP-HPLC)

Buffer A	
0.1 % (v/v)	TFA
in HPLC-grade H_2O	
Buffer B	
0.1 % (v/v)	TFA
in ACN	
Buffer C (storage buffer)	
65 %	ACN
35 %	HPLC-grade H_2O

2.2.1.5. Buffers for western blots

Transfer buffer	
50 mM	Tris
190 mM	Glycine
1 g/l	SDS
20 % (v/v)	Methanol
Blocking solution	
50 mM	Tris
190 mM	Glycine
1 g/l	SDS
20 % (v/v)	Methanol
pH 7.4	Adjusted by addition of HCL
PBS buffer	
4 mM	KH_2PO_4
16 mM	Na_2HPO_4
115 mM	NaCl
pH 7.4	Adjusted by addition of HCL
PBS-T buffer	
4 mM	KH_2PO_4
16 mM	Na_2HPO_4
115 mM	NaCl
0.1 % (v/v)	Tween 20
pH 7.4	Adjusted by addition of HCL
AP buffer	
100 mM	Tris base
100 mM	NaCl
5 mM	$MgCl_2$
pH 8.8	Adjusted by addition of HCL
NBT solution	
75 mg/ml	NBT
70 % (v/v)	DMF
Bcip solution	
60 mg/ml	Bcip
100 % (v/v)	DMF

2.2.1.6. Buffers for enzyme-linked immunosorbent (ELISA) Assay

Bicarbonate buffer	
50 mM	Na_2CO_3
50 mM	$NaHCO_3$
3 mM	NaN_3
pH 9.6	

PBS buffer	
1.5 mM	KH_2PO_4
8 mM	Na_2HPO_4
136 mM	NaCl
2.7 mM	KCl
pH 7.5	
PBS-T buffer	
1.5 mM	KH_2PO_4
8 mM	Na_2HPO_4
136 mM	NaCl
2.7 mM	KCl
0.05 % (v/v)	Tween 20
pH 7.5	
Diethanolamine buffer	
9.6 % (v/v)	Diethanolamine
1 mM	$MgCl_2$ x 6 H_2O

2.2.2. Media and agar

Media and agar were autoclaved before use. To avoid the formation of Maillard products, the sugar components were dissolved in an appropriate volume of water and autoclaved separately.

Malt extract agar	
2.0 % (w/v)	Malt extract
0.2 % (w/v)	Soy peptone
1.5 % (w/v)	Agar-Agar
pH 5.6	
YPG Agar and medium	
5 g/l	Yeast extract
10 g/l	Peptone
20 g/l	Glucose
15 g/l	Agar-Agar
pH 6.5	

2.2.3. **Organism, antibodies and peptides**

Microorganisms used for the infection of grapes or fermentation of grape juice are mentioned in the following. All microorganisms were stored as cryo cultures at -80 °C in the strain collection of the institute. Corresponding TMW numbers are mentioned for each of them.

Table 4: list of microorganisms used in the current work

Organism	TMW number	Source
Botrytis cinerea	TMW 4.2527	CBS 121.39, Centralbueau voor Schimmelcultures, Utrecht, Netherlands
Yeast 1 “bordeaux”	TMW 3.0704	Red wine starter, Paul Arauner GmbH & Co.Kg, Kitzingen, Germany
Yeast 2 “Bernkastel”	TMW 3.0705	White wine starter, Paul Arauner GmbH & Co.Kg, Kitzingen, Germany

Table 5: List of Antibodies and peptides used in the current work

Type	Description	Manufacturer
Antibody: Goat-anti-rabbit-IgG-AP	Anti-rabbit IgG (whole molecule) F(ab´)2 fragment- alkaline phosphatase antibody produced in goat	Sigma Aldrich, Saint Louis, MO, USA
Antibody: Anti-ns-LTP1-P2-IgG	Polyclonal rabbit antibody directed against a peptide of ns-LTP1 (pos. 62-86) from *Hordeum vulgare* (Specker, 2014)	ImmunoK, Amsbio, AMS Biotechnology, Oxfordshire, UK
Peptide: P2	Purity > 96 % 16 amino acids (pos. 62-86) from *Hordeum vulgare*	Davids Biotechnologie, Regensburg, Germany
Protein: ns-LTP1 (*Hordeum vulgare*)	Freeze dried supernatant of transgenic *Pichia pastoris* overexpressing ns-LTP1(Specker, 2014)	Technical University Munich, Freising, Germany

2.3. (Sparkling) wines, juices and grapes

In the following all juices, wines and sparkling wines, and grape samples used in the current work are listed. The sparkling wines as well as the base wines were anonymized before analysis. Therefore, not all information regarding manufacturer, grape variety or vintage can be provided.

Table 6: juice and grapes used in the current work

Product	Specification	Manufacturer
White grape juice “Eckes weißer Traubensaft”	pH 3.36, 60 °Oe MHD04.10.17 81 10:49 193	Eckes-Granini Deutschland GmbH, Nieder-Olm, Germany
Pinot blanc grapes	Collected in September 2014	Bavarian State Institute for Viticulture and Horticulture

Table 7: Bentonite treated Portugieser wines; vintage 2011 (wine number: 1141870, test number: W2-6110); provided by the Baverian State Institute for Viticulture and Horticulture, Veitshöchheim, Germany

Time of bentonite addition	Bentonite producer	Amount of bentonite added
-	-	0 g/hl bentonite
Fining of must	Producer A	300 g/hl bentonite
Fining of must	Producer B	300 g/hl bentonite
Addition after pre-clarification (Bentonite removal after fermentation)	Producer A	300 g/hl bentonite
Addition after pre-clarification (Bentonite removal after fermentation)	Producer B	300 g/hl bentonite
Fining of wine	Producer A	250 g/hl bentonite
Fining of wine	Producer B	250 g/hl bentonite

Table 8: Healthy and botrytized Pino blanc wines; provided by the Baverian State Institute for Viticulture and Horticulture, Veitshöchheim, Germany

Wine number	Grapes	Treatments
W3-14-14-1	healthy	-
W3-14-14-2	healthy	-
W3-14-14-3	healthy	-
W3-14-14- faul	botrytized	Sulfurization of the must and 100 g/hl charcoal

Table 9: ase wines and sparkling wines; details on grape manufacturing process or grape variety are anonymized

Type	Amount	source
White base wines	13 (El1-El13)	Verband deutscher Sektkellereien e.V. (VDS), Wiesbaden, Germany
Red base wines	8 (FR1-FR8)	Verband deutscher Sektkellereien e.V. (VDS), Wiesbaden, Germany
Sparkling wines	46	Directly from the manufacturer or obtained in a local supermarket

2.4. Software and databases

Software programs and databases used in the current work are listed in Table 1

Table 10: Software and Databases used in the current work

Program/Database	Application	Reference	Source
BLAST	Alignment of proteins and DNA	Wheeler et al. (2007)	https://blast.ncbi.nlm.nih.gov/Blast.cgi
Chromeolon^{TM} 6.80	Control, raw data storage and processing of chromatography experiments		Thermo Fisher Scientific Inc., Waltham, USA
Compute pI/Mw tool	Calculation of Mw and pI of proteins based on the amino acid sequence	Gasteiger et al. (2005)	http://web.expasy.org/compute_pi/
Flexanalysis 3.3	Visualization of spectra obtained by MALDI-TOF MS		Bruker Daltonics GmbH, Bremen, Germany
InterPro v.60	Functional analysis of proteins	Mitchell et al. (2014)	https://www.ebi.ac.uk/interpro/
MALDI Biotyper 2.0	Controlling of the MALDI-TOF MS		Bruker Daltonics GmbH, Bremen, Germany
Mascot 2.3 02	Identification of proteins	Koenig et al. (2008)	Matrix Science, London, UK
Protein Prophet	Statistical model for identifying proteins	Nesvizhskii et al. (2003)	Institute for Systems Biology, Seattle, WA, USA
Scaffold 4.3.4	Visualization and validation of complex MS/MS proteomics experiments	Searle (2010)	Portland, OR, USA

Program/Database	Application	Reference	Source
SignalP 4.1	Prediction of signal cleavage sites in amino acid sequences	Petersen et al. (2011)	http://www.cbs.dtu.dk/services/SignalP/
UniProt database	Protein database	UniProtConsortium (2017)	http://www.uniprot.org
X! Tandem	Matching mass sepctra with peptide sequences	Bjornson et al. (2007)	http://www.thegpm.org/tandem/
Yaspin	Prediction of protein secondary structure	Lin et al. (2005)	http://www.ibi.vu.nl/programs/yaspinwww/

2.5. Microbiological methods

2.5.1. *B. cinerea* and *S. cerevisiae* cultures

B. cinerea (see Table 4) was grown on malt extract agar plates (3 % w/v malt extract, 0.3 % w/v soy peptone, pH 5.2). Fungal conidia were harvested mechanically using a sterile Drigalski spatula and sterile deionized water. The density was adjusted to 6×10^7 conidia/ml using a hemocytometer (Thoma type, 0.1 mm chamber depth). For the fermentation of the grape juice precultures, two different *S. cerevisiae* strains were prepared. A colony of the yeast strain TMW 3.0704 termed yeast 1, and the yeast strain TMW 3.0705, termed yeast 2, were inoculated directly from YPG agar plates into YPG broth and incubated for two days at ambient temperature (approximately 23°C) under diffused daylight (for a detailed description of the yeast strains see Table 4). The viable cell concentrations were counted using methylene blue for life-dead staining and the hemocytometer mentioned previously. Cultures were adjusted to 2×10^8 living cells/ml.

2.5.2. Infection of grapes

Grapes of the Pinot blanc variety were collected in September 2014 at a vineyard in Veitshöchheim (Germany). The infection of the grapes in the laboratory was conducted based on the protocol of Girbau et al. (2004) with some modifications. For surface sterilization, the grapes were treated once by immersion for 5 min in 70 % ethanol followed by two treatments with 6 % sodium hypochlorite and subsequently rinsed three times with sterile deionized water. Infection of the grapes was carried out by pricking berries with a sterile tooth pick previously immersed in a solution of 4×10^6 conidia/ml of *B. cinerea* (see Table 4) in 0.1 % Triton X-100. Each berry was incubated separately at ambient temperature in the wells

of a sterile 24-well plate. The control berries were treated in the same way using deionized water with 0.1 % Triton X-100 for punctuation. After incubation for 8 d at ambient temperature (approximately 23 °C) in diffused day light all grapes from a 24-well plate were separately collected and mashed. Mashed berries were frozen at -20 °C and subsequently freeze-dried under vacuum. Freeze dried samples were stored at -20 °C until further processing.

2.5.3. **Fermentation of grape juice**

For the fermentation experiments, white untreated grape juice (specification see Table 6) was obtained from a local supermarket and filtered through a 0.2 µm sterile filter. According to the manufacturer's data, the grape juice was composed of 100 % pure juice without the addition of preservatives. No data were available on heat and bentonite treatment of the juice. A previous heat or bentonite treatment does not influence the results of the fermentation experiments. All experiments were performed from the same production batch of grape juice for an optimal comparability.

An amount of 400 ml of the filtered juice was filled into autoclaved 500 ml Erlenmeyer flasks. Altogether, 18 Erlenmeyer flasks were prepared in this way. To investigate the influence of a preliminary *B. cinerea* infection, half of the samples was inoculated with 200 µl of the prepared conidial solution (see section 2.5.1) and termed "botrytized samples". The other half of the samples was not infected with *B. cinerea* and was termed "healthy samples". The infected samples as well as the uninfected samples were incubated for 1 week under diffused daylight at ambient temperature (approximately 23 °C). Three healthy and three infected juice samples were treated with potassium metabisulfite to a final concentration of 100 mg/l respectively. The manufacturer recommends adding 100 mg/l to must if microbiological contaminations are present. The amount of free sulfite was not determined in the samples but was calculated from the manufacturer's data. The product decomposes and liberates around 50 % of free sulfite. Consequently, 50 mg/l free sulfite was available in each flask. Three other healthy and infected samples were heat-treated for 5 min at 85°C before yeast addition. A volume of 10 ml of the yeast 1 and yeast 2 precultures (see section 2.5.1) was added respectively to the infected and healthy sulfur- and heat-treated juice samples as described above. After a further incubation for 10 days at ambient temperature (approximately 23 °C) under diffused daylight, the fungal mycelium was separated by filtration through a muslin bandage. The residual yeast cells were removed by centrifugation

of the samples in sterile Falcon tubes for 5 min at 5,000 × g. Before further processing, the samples were dialyzed against 20 times their volume of deionized water for 72 h in dialysis tubes (MWCO 3500). The experiment was performed in biological and technical duplicates. Protein purification for reversed-phase high-performance liquid chromatography (RP-HPLC) as well as for SDS-PAGE analysis was performed as described in the following.

2.6. Protein chemical methods

2.6.1. **Samples preparation dialysis and freeze-drying**

To remove low molecular weight interfering substances, the juice, wines, and sparkling wines were dialyzed against 20 times their volume of deionized water for 72 h at 4°C to prevent protein degradation. 50 ml or 10 ml of the samples were filled in dialysis tubes (3.5 kDa cut off) respectively and the water was replaced every 12 hours. After 3 days, the samples were transferred into 250 ml round-bottom flasks and frozen at -20°C. If a volume of 10 ml was dialyzed the samples were transferred into a 50 ml Falcon tube before freezing. The subsequent freeze-drying was carried out overnight. Until further processing the lyophilizates were stored at -20°C.

2.6.2. **Protein purification**

For protein purification the protocol as described in Vogt et al. (2016) was applied. All buffers used are listed in section 2.2.1.1. Before protein purification from juice, wine ore sparkling wine was carried out, the samples were dialyzed and freeze dried as described in chapter 2.6.1. All working steps were performed on ice and the centrifugation steps at 4 °C. For SDS-PAGE analysis an initial volume of 10 ml of (sparkling) wine was applied. 50 ml (sparkling) wine was used to purify proteins for RP-HPLC or ELISA. The lyophilisates were dissolved in 1 ml (initial sample volume 50 ml) or in 800 µl (initial sample volume 10 ml) buffer B1 and centrifuged for 5 min at 5,000 x g. Since protein purification was carried out in technical duplicates, the dissolved lyophilisates were split equally to 490 µl (dissolved in 1 ml buffer B1) or 390 µl (dissolved in 800 µl buffer B1) respectively and transferred into reaction tubes. For the removal of phenolic compounds, an equal amount of water saturated phenol was added (390 µl and 490 µl respectively) and mixed for 30 min at 4 °C. After centrifugation for 15 min at 6,000 x g the upper aqueous phase was discarded. The removal of interfering compounds by the addition of buffer B1 was done two times. For protein precipitation, buffer B2 was added to the remaining phase. 1 ml buffer B2 was added if

protein purification was carried out from an initial volume of 10 ml and 1.5 ml buffer B2 was added if an initial volume of 50 ml was assessed. After vortexing, the proteins were precipitated overnight at -20 °C, centrifuged for 40 min at 40,000 x g and the supernatant was discarded. The protein pellet was washed by the addition of 1.5 ml buffer B3 and a following incubation for 60 – 90 min with a subsequent centrifugation for 30 min at 13,000 x g. To remove the ammonium acetate, the washing step was repeated using buffer B4. The washed and dried protein pellets were stored at -20°C until further processing. For the purification of proteins from grapes, the following modifications on the extraction buffer were made to exclude solid substances from grape samples. 100 mg of the lyophilized mashed grapes were dissolved in extraction buffer 1_G without KCl but containing additionally 0.8 M sucrose to result in a higher density of the aqueous phase. After the first centrifugation step the upper phenolic phase was recovered, whereas the insoluble substances of the grapes were discarded with the lower aqueous phase. Further processing of the phenolic phase from mashed grapes was carried out as for the phenolic phase of (sparkling) wines.

2.6.3. SDS-PAGE

For the comparison of the electrophoretic patterns of grapes, juices, wines and sparkling wines or to check the success of protein isolation, SDS-PAGE analysis was carried out. Since the current work was focusing on the low molecular weight proteins (< 20 kDa), SDS-PAGE analysis was carried out according to the protocol of Schägger and Von Jagow (1987) using a 16 % polyacrylamide separating gel. The buffers and the composition of the stacking and separating gel are listed in section 2.2.1.2. The separating and stacking gel were casted successively between two glass plates using a 1 mm spacer. Polymerization of the acrylamide was induced by the addition of TEMED and APS immediately prior to casting. To ensure a clear separation line between the separating and stacking gel, the separating gel was overlaid with 100 % isopropanol till the polymerization process was completed. Before the addition of the stacking gel, the isopropanol was removed. Storage of the gels was possible up to one week in humid tissues at 4 °C.

Protein pellets (protein purification; see section 2.6.2) were dissolved in 15 µl application buffer. If not otherwise mentioned 7.5 µl of the sample were mixed with the same amount of deionized water. Lyophilizates were dissolved in deionized water and subsequently diluted with the same amount of application buffer. Protein denaturation was achieved by heating up the samples to 95 °C for 5 min immediately prior to SDS-PAGE analysis. For each run, a

final volume of 10 µl was applied into the gel pockets. Electrophoresis was started with a constant voltage of 80 V for 10 min and then raised to 100 V for about 110 min until the dye front reached the end of the gel.

2.6.4. **Silver staining**

Visualization of the proteins after SDS-PAGE analysis was carried out by silver staining according to Blum et al. (1987). The procedures for the different staining steps are listed below. The solutions were prepared as mentioned in section 2.2.1.3. Developing of the gels was stopped when the protein bands became visible. Different gels that were compared in the following regarding their protein amount were developed at the same time.

Step	Solution	Duration
Fixation	Fixative	> 3 or overnight
Washing	Wash solution	2 x 20 min
Washing	dH_2O	20 min
Sensitization	Thiosulfate reagent	1 min
Washing	dH_2O	3 x 20 s
Silver nitrate	Silver nitrate reagent	20 min
Washing	dH_2O	3 x 20 s
Developing	Developer	up to 5 min
Washing	dH_2O	3 x 20 s
Stopping	Stop reagent	5 min

2.6.5. **Identification of proteins**

For protein identification, the proteins were applied on an SDS-PAGE as described in section 2.6.3. and subsequently silver stained using the FireSilver Staining Kit according to the manufacturer's guidelines. For identification, protein bands were excised from the stained gel and sent to the Protein Analysis Unit at Ludwig-Maximilians-Universität München (LMU, Munich, Germany) for in-gel digestion using trypsin and subsequent nano-ESI-LC-MS/MS analysis. Proteins were identified by the Protein Analysis Unit using Mascot and X! Tandem (The GPM, thegpm.org; version CYCLONE (2010.12.01.1)) to correlate spectra with entries in the UniProt database (*Vitis vinifera*, ID 29760; *Botrytis cinerea*, ID 999810; *Aspergillus niger*, ID 425011; *Penicillium chrysogenum,* ID 500485; *Saccharomyces cerevisiae,* ID 559292) using Scaffold. Peptide identifications were accepted if they could be established at < 95.0 % probability by the Scaffold Local FDR algorithm. Protein identifications were accepted if they could be established at < 99.0 % probability and contained at least 2

identified peptides. Protein probabilities were assigned by the Protein Prophet algorithm. Functional analysis of the identified proteins was carried out using InterPro v.60. Signal peptides were predicted using SignalIP 4.1 server.

2.6.6. **Western blot analysis**

Western blot analysis was carried out to detect ns-LTP1 from *V. vinifera* in grapes. The proteins separated by SDS-PAGE analysis were transferred from the gel to a PVDF membrane by applying voltage. The protein of interest was detected using antibodies linked with an enzymatic color reaction (Matsudaira, 1987). All buffers and solutions are listed in section 2.2.1.5. The proteins of the grapes were purified as described in section 2.6.2 and a subsequent SDS-PAGE analysis was carried out (see section 2.6.3). Prior to the blotting process the PVDF membrane was wetted in 100 % Methanol. The gel, as well as the blotting papers were equilibrated for 20 min in transfer buffer. For the protein transfer 3 filter papers were layered followed by the gel, the PVDF membrane and another 3 filter papers. Air bubbles between the single layers were avoided to enable a trouble-free protein transmission. The PVDF membrane was orientated on the anode side and all the layers were subjected to constant voltage of 75 mA per gel for 50 min in the semi-try blotter. The proteins migrated in the electric field from the cathode to the anode due to their negative charge. The PVDF membrane was blocked by swirling it carefully in blocking solution overnight at 4 °C and subsequently washed three times with PBS-T buffer for 10 min. The primary antibody directed against ns-LTP1 (detailed specifications see Table 5) was diluted (1:2000) in PBS buffer. The PVDF membrane was incubated for 1.5 h at ambient temperature with 25 ml of the antibody solution followed by another 3 washing steps with PBS-T buffer for 10 min respectively. In the following the membrane was incubated for another 1.5 h in a PBS solution containing the secondary antibody (detailed specification see Table 5) diluted 1:5000 in PBS buffer. To prevent unspecific binding of the antibody to the PVDF membrane, it was washed twice with PBS-T buffer and with PBS buffer for 5 min, respectively. Before starting the enzymatic color reaction, the membrane was equilibrated for 5 min in AP buffer and subsequently incubated in 15 ml AP buffer containing 7.5 µl NBT solution and 15 µl Bcip solution. The staining of the membrane was stopped by transferring it to deionized water.

2.6.7. **MALDI-TOF MS analysis**

The matrix-assisted desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) is a powerful tool for protein analysis. The samples get co-crystallized in an organic

matrix that adsorbs at a specific wavelength. Laser treatment results in a protonation of the analytes accompanied by a desorption into the gas phase. The m/z ratio is determined in a TOF mass analyzer. In the current work, sinapinic acid was used as matrix in a concentration of 10 mg/ml dissolved in an organic solvent containing 50 % ACN and 2.5 % TFA in deionized water. Sample application was carried out using the double layer method. The target was coated with 1 µl of the matrix and after air drying 1 µl of the sample was applied and dried before covering with another 1 µl of matrix and drying. Each mass spectra were composed of 240 single mass spectra obtained by laser shots (λ = 337 nm).

2.6.8. **Protein deglycosylation**

Deglycosylation of proteins using triflouromethanesulfonic acid was performed according to the manufacturer's guidelines with some modifications. After neutralization with pyridine the proteins were precipitated over night with 2 ml 0.1 M ammonium acetate in methanol. The resulting protein pellet was separated from the supernatant by centrifugation (40 min, 17,000 x g, 4 °C) and washed with 80 % acetone.

2.6.9. **Reversed-phase high performance liquid chromotography**

2.6.9.1. Quantitative RP-HPLC analysis

The buffers used for reversed-phase high-performance liquid chromatography (RP-HPLC) are listed in section 2.2.1.4. Analytes were eluted from the column in a linear gradient from buffer A to buffer B with a flow rate of 0.4 ml/min. Each measurement started with 3 % buffer B followed by a linear gradient to 35 % buffer B within 35 min. The eluents were detected at 214 nm. Considering the signal-to-noise ratio, the detection limit was set at 5 mAU*min. When analyzing the purified proteins (see section 2.6.2), the pellets were dissolved in 50 µl 0.1 M NaOH followed by a stepwise addition of 50 µl 8 M Urea, 12 µl acetonitrile and 288 µl 0.1 % trifluoracetic acid in ddH_2O. The samples were filtrated trough a 0.2 µm filter to remove insoluble substances before injection. A quantitative evaluation of samples was conducted by comparing the peak areas of the peaks of interest using an injection volume of 20 µl.

2.6.9.2. Preparative RP-HPLC analysis

For the isolation of PAU5 in its native state, a method for protein isolation via RP-HPLC was developed as follows: A bottle of white non-gushing sparkling wine was obtained from a

local supermarket, dialyzed against 20 times its volume of deionized water for 72 h in dialysis tubes as mentioned previously and freeze dried. The freeze-dried sparkling wine was dissolved in 1 ml HPLC buffer containing 15 % v/v acetonitrile and 0.1 % v/v trifluoracetic acid in deionized water and centrifuged for 10 min at 20,000 × g at 4 °C to separate insoluble substances. The supernatant was applied onto RP-HPLC using an injection volume of 80 µl. Each run started with a concentration of 85 % buffer A (0.1 % v/v trifluoracetic acid in ddH$_2$O) and 15 % buffer B (0.1 % v/v trifluoracetic acid in acetonitrile) with a constant flow rate of 0.4 ml/min. After 5 min, the acetonitrile concentration was raised to 35 %. For the elution of PAU5, a linear gradient from 35 % to 46 % acetonitrile within 5 min was conducted. Under the set experimental conditions, PAU5 eluted at an acetonitrile concentration of 41 – 45 %. For protein isolation, the eluate from each run in the range of the above mentioned acetonitrile concentration was collected. This experiment was repeated until all the supernatant was applied to RP-HPLC. The eluates were pooled and dialyzed overnight against 20 volumes of deionized water in dialysis tubes as before and subsequently freeze dried as described in section 2.6.1.

2.6.10. **ELISA Assay**

For detection and quantitative comparison of ns-LTP1 amounts in sparkling wines, wines and juices, a competitive ELISA was developed. Peptides and antibodies used are listed in Table 5. Each measurement was carried out in triplicates. The composition of the buffers can be found in section 2.2.1.6. Wine and sparkling wine samples were dialyzed and freeze-dried as described in section 2.6.1. For coating of the microtiter plates 15 ng/ml of the peptide ns-LTP1-P2 was dissolved in Bicarbonate buffer and 100 µl was pipetted into each well. Incubation was carried out over night at 4 °C. Each well was washed 3 times with 200 µl PBS-T buffer respectively. If not otherwise mentioned the sample was dissolved in 330 µl PBS buffer and 100 µl per well was applied (initial volume: 10 ml per well), hence the measurement was carried out in technical triplicates. For the determination of the blank, PBS buffer was added without any sample in 3 of the wells. As a positive control 10 mg/ml of the freeze-dried supernatant of transgenic *Pichia pastoris* overexpressing ns-LTP1 (specification see Table 5) was applied in PBS buffer. The primary antibody directed against ns-LTP1 was diluted 1:10 in PBS buffer and 3 µl were added to each well. After an incubation of 1.5 h at ambient temperature the wells were washed 3 times with 200 µl PBS-T buffer respectively. The second antibody was diluted 1:5000 in PBS buffer and 100 µl were applied in each well and incubated for 30 min at ambient temperature. Each well was washed 3 times with PBS-T

buffer and 2 times with PBS buffer. 0.5 mg/ml pNPP was dissolved in diethanolamine buffer and 100 µl per well of the solution were incubated for 30 min at ambient temperature. The enzymatic color reaction was determined by measuring the extinction at 405 nm. Hence a competitive ELISA was applied the measured signal was inversely proportional to the concentration of ns-LTP1 in the sample. For a comparison of the single values the relative amount was calculated as described in the following equation.

$$rel.intensity\ [\%] = \left(100 - \frac{M}{A} \times 100\right) + (100 - \frac{B}{A} \times 100)$$

M: Mean absorbance value of the sample
A: Mean absorbance value of the positive control
B: Mean absorbance of blank

2.7. Analysis of foam stability

2.7.1. Foam stability analysis of base wines

The analysis of the foaming properties was performed by the perfusion of samples with a constant nitrogen flow. The lower end of a vertical polyacrylamide column with an inner diameter of 1.5 cm was placed on a porous glass frit connected to a nitrogen supply. For analysis, samples were filled into the column to a height of 7 cm (37.5 ml of sample). Nitrogen was perfused at a constant flow with the result that the foam in the column reached its maximum height of 28 cm. For comparison of the foam stability, the time of the foam decay after stopping the nitrogen flow was monitored. Each measurement was performed in technical duplicates. For the determination of the decay rate, the nitrogen flow was stopped after 5 min and the time for the foam decay as well as the maximum foam height was recorded. The decay rate was defined as the ratio of maximum foam height to the time needed for foam decay.

2.7.2. Foam stability analysis of the protein PAU5

The purified and freeze-dried protein PAU5 (3.5 mg) was dissolved in 200 µl 0.5 M Tris buffer (pH 7.5) and split into two 100 µl fractions with a final concentration of 17.5 mg/ml. One of those protein fractions was treated with 5 mg proteinase K to degrade the protein

PAU5 overnight at 37°C. To eliminate insoluble protein precipitates occurring during protein digestion, the samples were centrifuged for 5 min at 5,000 × g before analysis.The success of the protein digestion was checked by analysis of 3 µl of each fraction by SDS-PAGE as previously described in section 2.6.3. The measurement of the foam stability was conducted using untreated white grape juice bought in a local supermarket. The addition of PAU5 to grape juice (37.5 ml) was performed by a stepwise addition of the dissolved protein. The foam stability of the juice containing the protein with final volumes of 40 µl, 60 µl, and 70 µl, respectively, was measured. Consequently, the final concentrations of PAU5 in the grape juice were 18.67×10^{-3} mg/ml, 28.00×10^{-3} mg/ml and 32.67×10^{-3} mg/ml. The protein that had previously been digested with proteinase K was added in the same volumes to 37.5 ml of untreated white grape juice with a final concentration of proteinase K of 53.33×10^{-3} mg/ml, 80.00×10^{-3} mg/ml and 93.33×10^{-3} mg/ml, respectively. To determine the effect of the proteinase K on the foam stability, 5 mg of this enzyme was dissolved in 100 µl 0.5 M Tris buffer (pH 7.5) and heated up to 85°C for 5 min for inactivation of the enzymatic activity. Then, it was added to the grape juice in the same manner as PAU5 and the digested protein.

3. RESULTS

3.1. Types of over-foaming

For the comparison of the foaming behavior, the sparkling wines were left unmoved over night at ambient temperature and subsequently opened. In general, the bottles affected by the phenomenon gushing that were opened in the laboratory could be divided into two groups according to their foaming behavior. Some of the bottles showed a slow release of big bubbles over a long period of time (see Figure 4 A). A closer examination of the bubble formation revealed that the bubbles causing the over-foaming grew only in certain locations inside the bottle. Bubble formation happened either on the wall of the bottle or, in most of the cases, on the bottom of the bottle suggesting nucleation sites in this area to be responsible for bubble growth. This type of over-foaming was defined as Type I.

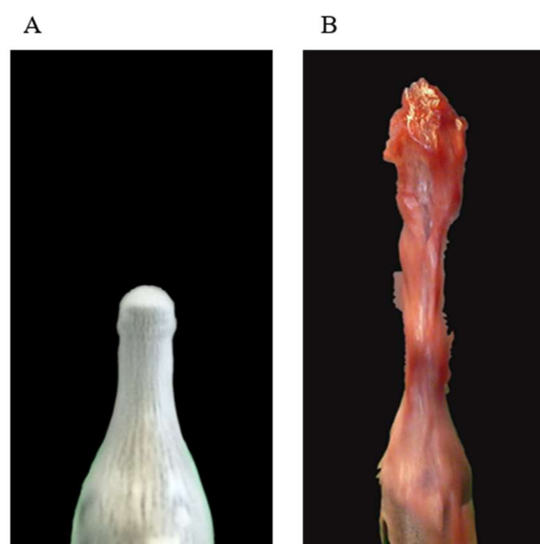


Figure 4: Gushing of sparkling wines immediately after opening; A) Type I of gushing described in the text; B) Type II of gushing described in the text

The other type of gushing, named Type II over-foaming, was characterized by a massive but quick over-foaming after pressure release with small bubbles (see Figure 4 B). Here, the bubbles arose immediately after opening of a bottle simultaneously in the liquid suggesting that nucleation sites can be found equally distributed all over the volume of the liquid. This kind of over-foaming was in general accompanied by a high loss of volume. Sparkling wine samples differed in their intensity of over-foaming (from 2 ml to 500 ml). Additionally, there were also gushing sparkling wines containing characteristics of both over-foaming types and showed temperature dependence in their over-foaming behavior. Therefore, a clear

classification of sparkling wines regarding to their over-foaming characteristics was not always possible. Nevertheless, those first observations showed that gushing in sparkling wines might be caused by different inducers like the gushing inducing types in beer. Solid particles that were found in some of the gushing sparkling wines were potentially gushing inducing and will be discussed in the following chapter suggesting secondary gushing. In addition, the protein composition of gushing and non-gushing sparkling wines will be compared in order to find surface-active proteins influencing the gushing potential, suggesting that they might be involved in primary gushing of sparkling wines.

3.2. Particles in gushing grape derived beverages

3.2.1. Crystals in gushing sparkling wines

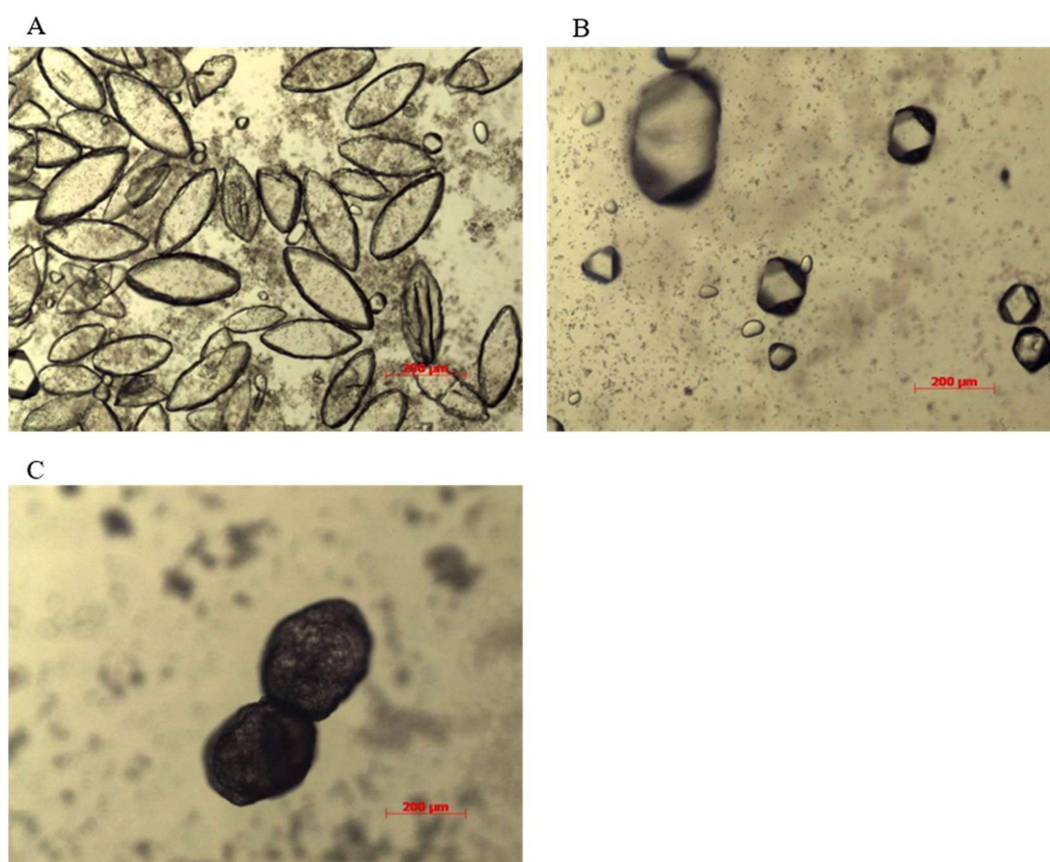


Figure 5: Types of crystals found in gushing sparkling wine prior to disgorging according to the manufactures data (100-fold magnification); A) no over-faoming after opening in the laboratory; B) 210 ml over-foaming; C) 100 ml over-faoming

In some of the gushing sparkling wines, crystals were found on the bottom of the bottle. According to manufacturers, all these sparkling wines showed gushing behavior prior to disgorging. Most of them were classified as Type I gushing as described previously

suggesting that some of the crystals act as nucleation sites. Indeed, the majority of the bubbles grew on some of the crystals immediatley after opening. But not all sparkling wines containing crystals did show gushing behavior. The crystals found in the gushing sparkling wines differed in size, shape and appearance. Three samples discribed in the following were analyzed before disgorging. In one sparkling wine the crystals were found to be clear with an oval shape and a flat surface (Figure 5 A). This sample did not show any gushing behavior after opening of the bottle at ambient temperature in the laboratory but was provided as a sample with advers foaming properties according to the manufacturers data. Another sample showed a Type I overfaoming volume of 210 ml after opening in the laboratory. The crystals found on the bottom of the bottle had a more complex shape (Figure 5 B). The third type of crystals was found in a sparkling wine with Type I overfoaming of 100 ml. They showed a rough surface partially covered with yeast cells (Figure 5 C). Hence the thesis is focussing on the role of proteins in the development of gushing, no further investigations on the character of the crystals was carried out. The type of crystals and their formation in sparkling wine as well as parallels to crystals found in beer and the development of gushing will be discussed later in the text..

3.2.2. **Residual filter aids in gushing carbonated grape juice**

A carbonated red grape juice showed massive Type II over-foaming after opening of the bottle at ambient temperature. This sample showed a highly temperature-dependent gushing behavior. Opening of the bottle before cooling down to 4 °C led to less volume loss and an over-foaming as described for Type I suggesting that in the chilled sample nucleation sites accumulated on the bottom of the bottle. No visible crystals were found in the sample. However, centrifugation of 50 ml at 5,000 x g for 20 min resulted in the sedimentation of small particles but not in the formation of a pellet. Even an increase of the centrifugation time and power did not result in pellet formation.

Microscopic characterization of the particles revealed that the sediment consisted of small particles. They were about tenfold smaller than the crystals found in gushing sparkling wines mentioned previously and did not show any crystal formation. The particles differed in their appearance and size and looked rather like small fibers (Figure 6A). They were assumed to act as nucleation sites for CO_2 in this case of gushing. The microscopic picture was compared to images found in the literature dealing with gushing in beer. A visual examination revealed that the particles found in gushing carbonated grape juice were similar in shape as

microscopic images of filter aids found to be a gushing inducer in beer, e.g. charcoal, perlites or diatomaceous earth (Figure 6 B - E) (Gastl & Zarnkow, 2009). Therefore, it was suggested that in this case gushing was induced by residual filter aids.

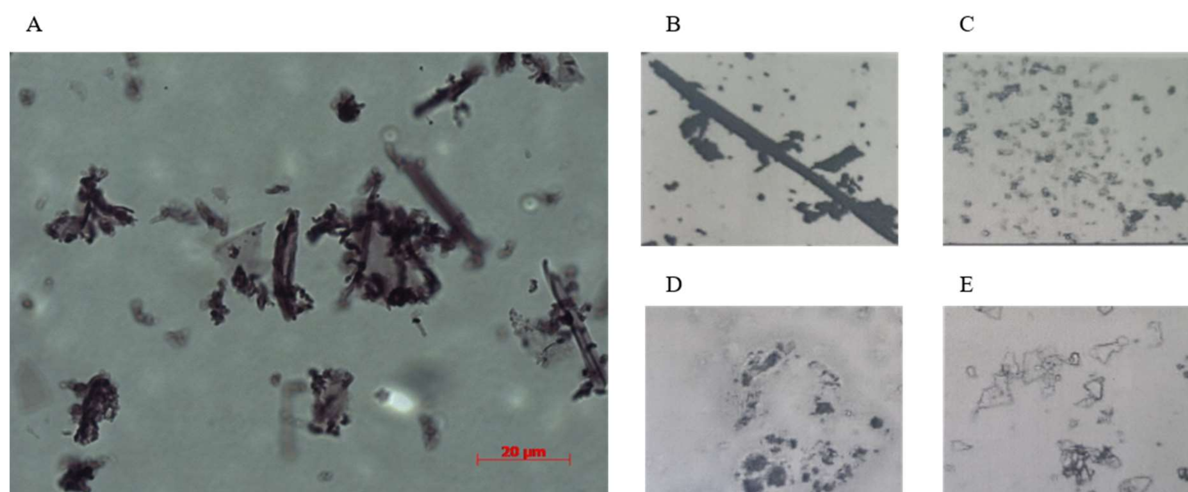


Figure 6: (A) microscopic image of particles found in gushing carbonated grape juice, 1000-fold imagination; (B) charcoal, (C)+(D) and (E) perlite found in beer as gushing inducing substances, 400-fold imagination (Zarnkow & Back, 2001)

3.2.3. Protein haze in gushing sparkling wine

Two different samples of sparkling wines of similar make were provided to the current study by a manufacturer. One of them did not show any gushing behavior, the other was a gushing sparkling wine with a massive Type II over-foaming at ambient temperature suggesting that the nucleation sites were distributed all over the liquid.

The gushing sparkling wine was analyzed for the presence of small crystals or particles without success. During further analysis of the sparkling wines in test tubes it turned out that the gushing sparkling wine differed in its light scattering properties from the non-gushing negative control (Figure 7). This effect is known as the Tyndall-effect and described as light scattering induced by small insoluble particles in a very fine suspension. An additional analysis measuring the optical density of 600 nm confirmed this observation. The optical density was about 2 times higher in the gushing sparkling wine compared to the negative control.

For a more detailed investigation of those small insoluble particles, 50 ml of the sparkling wines were centrifuged for 1 h at 10.000 x g. The centrifugation of the gushing sparkling wine resulted in the formation of a sediment, whereas in the non-gushing sparkling wine no

visible sedimentation occurred after centrifugation. The sediment was washed two times with deionized water and centrifuged again under the conditions described above.

Figure 7: sparkling wines in test tubes, left: gushing sparkling wine with modified light scattering properties, right: non-gushing sparkling wine as negative control

A further analysis using a microscope at 1000-fold magnification showed that the particles were amorphous rather than crystalline which suggested that they consist of protein aggregates. To verify this observation, the washed sediment was dissolved in 30 µl of application buffer. Furthermore, 3 µl of the sample were applied to SDS-PAGE with subsequent silver staining. SDS-PAGE analysis revealed that the sediment found after centrifugation of the gushing sparkling wine consisted of proteins with a molecular weight smaller than 26 kDa (Figure 8). The conditions present in the sparkling wine may have led to the formation of insoluble aggregates consisting of those small proteins inducing the Tyndall-effect. Therefore, it was assumed that protein aggregates acted as nucleation sites after opening the bottle and were thus responsible for the massive over-foaming in this case.

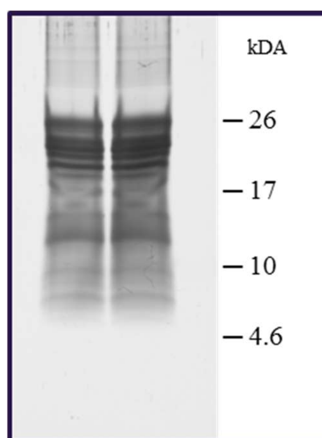


Figure 8: SDS-PAGE analysis of the sediment of protein aggregates analyzed in duplicates of the gushing sparkling wine with modified light scattering properties, Proteins are visualized by silver staining.

3.3. Method development for protein analysis

3.3.1. Method development for protein purification and SDS-PAGE analysis

The current thesis is focusing on solubilized surface-active proteins in sparkling wine and their influence on the gushing potential and foaming behavior. For proper analysis of wine and sparkling wine, appropriate methods needed to be selected and developed. SDS-PAGE analysis, a common tool for protein analysis, is susceptible to interfering substances such as polyphenols. Some of them are covalently bound to proteins in (sparkling) wines via disulfide bridges that make protein purification, especially in red (sparkling) wines, more challenging. So far, only two previous publications have addressed the analysis of the protein composition in red wine by SDS-PAGE (D'Amato et al., 2010; Wigand et al., 2009). Both authors tried to decrease the concentration of phenolic compounds using PVP(P). This protein purification strategy was also applied during the current work. A treatment with PVP of 3 mg dialyzed and freeze dried red sparkling wine resulted in significant reduction of the overall protein content. The protein bands were hardly visible and no single bands were detectable after silver staining (data not shown). Therefore, an analysis via SDS-PAGE was not possible using the previously published protocols.

Consequently, a new strategy for protein purification of red (sparkling) wines had to be developed during the current study. The purification strategies described in the following were carried out with the same sample of red sparkling wine to enable the best possible comparison of results. To control the efficiency of the purification techniques, an SDS-PAGE with subsequent silver staining was carried out applying the same amount of sample. All

samples were dissolved in 15 µl application buffer and 8 µl of dissolved sample were diluted with 7 µl dH_20. A final volume of 10 µl was assessed by SDS-PAGE.

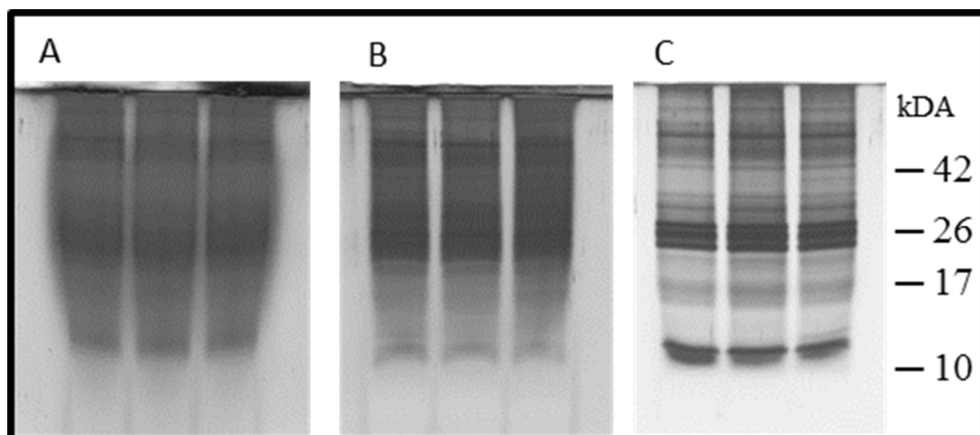


Figure 9: SDS-PAGE of red sparkling wine with three different preparation techniques for the sample. Proteins are visualized by silver staining. (A) Method A: Dialysis and lyophilisation of the red sparkling wine. (B) Method B: Protein precipitation with ammonium acetate after method A. (C) Method C: Combination of method B with extraction of phenols by the use of water-saturated phenol. Proteins are visualized by silver staining

Each purification approach was carried out in triplicates. For the removal of small interfering substances like sugars, ethanol or polyphenols that are not interacting with proteins, the red sparkling wine was dialyzed (3.5 kDa cut off) against dH_20 and subsequently freeze-dried to achieve a higher concentration of proteins in the sample. Three mg of the lyophilized sample was dissolved as described above and directly applied to SDS-PAGE (Figure 9A). The high amount of phenolic substance left in the sample resulted in a high background. It was not possible to distinguish particular bands in the sample.

In the next purification approach three mg dialyzed and freeze-dried red sparkling wine was dissolved in 300 µl of the reducing aqueous buffer B1 in order to cleave disulfide bonds between proteins and phenolic compounds. To achieve a higher concentration of the proteins, they were precipitated over night at –20 °C using 900 µl of 0.1 M ammonium acetate in methanol and centrifuged for 10 min at 3,000 x g at 4 °C. The resulting pellet was dissolved in application buffer and applied to SDS-PAGE (Figure 9B). The protein precipitation using ammonium acetate resulted in a reduction of the background but there were still no single protein bands detectable concluding that a high amount of interfering substances still remained in the sample.

Therefore, a new and more complex method was developed and optimized to remove interfering substances. The protein purification was based on the protocol of Hurkman and Tanaka (1986). The samples were dialyzed and freeze dried. The reducing aqueous buffer B1

was applied to three mg of the lyophilizate and the same amount of water-saturated phenol was added. The proteins remained in the lower phenolic phase whereas the upper aqueous phase was discarded. Protein precipitation was carried out using 0.1 M ammonium acetate in methanol. Before further analysis, the protein pellet was washed with methanol and acetone, respectively (as briefly described in section 2.6.2). During the optimization process several numbers of washing steps with the aqueous buffer were tried as well as different solvents instead of water-saturated phenol were tested. Best results were obtained by carrying out two washing steps using water-saturated phenol. A subsequent SDS-PAGE analysis with silver staining resulted in clearly resolved protein bands with a considerably reduced background (Figure 9 C). Although the modified purification protocol included several steps, it showed highly reproducible results and a strongly improved resolution. As depicted in Figure 9, there was no visible difference in the electrophoretic pattern between technical triplicates.

To elucidate the applicability of the developed method, it was implemented on a white sparkling wine, a Rosé wine as well as red grape juice. A subsequent SDS-PAGE and silver staining of gels showed in all three cases protein bands that were clearly distinguishable from each other with a good resolution and a low background (Figure 10 A - C). Consequently, the developed method is a suitable tool for protein purification for all kind of grape derived beverages.

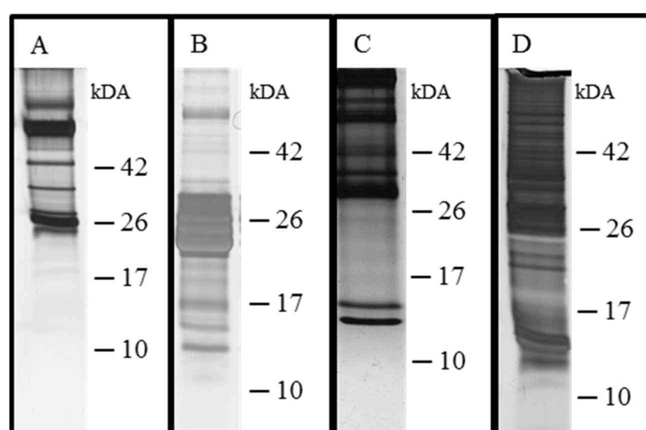


Figure 10: Application of the new method C for (A) white sparkling wine, (B) rosé wine Weißherbst, (C) red grape juice, (D) Pinot noir grapes treated with the modified protocol (sucrose added to B1), Proteins are visualized by silver staining

In the following, the new technique was conferred to the protein analysis of grapes. In contrast to the grape derived beverages, a large quantity of the grapes consists of insoluble particles. Consequently, it was necessary to implement a strategy for the removal of those insoluble particles within the protein purification process. The grapes were freeze-dried,

smashed and 0.05 g was resolved in the reducing aqueous buffer B1 containing sucrose. The addition of sugar to the buffer resulted in an altered density of the aqueous phase. After centrifugation, the upper phenolic phase was recovered whereas the insoluble substances from the grapes remained in the lower aqueous phase. The following steps of purification were carried out in the same way as the purification process described for sparkling wines. Considering the high amount of protein in the grapes, the obtained protein pellets were dissolved in 100 µl application buffer. For SDS-PAGE analysis, 1.5 µl of the sample was mixed with 6 µl application buffer and 7.5 µl dH_20. A final volume of 10 µl was applied to SDS-PAGE with subsequent silver staining (Figure 10 D). Protein bands with a molecular weight below 26 kDa were clearly separated from each other and showed a high resolution. The diminished resolution of higher molecular weight proteins was rather due to the high diversity of proteins than to an interfering background. Results showed that the new method can be applied with some modifications also for protein purification from grapes.

3.3.2. **Method development for RP-HPLC**

For a quantitative analysis of small proteins and peptides in grape derived beverages a protocol for RP-HPLC was developed. The RP-HPLC was equipped with a UV-Detector measuring the absorbance of the eluting sample at 214 nm. As some phenolic compounds might interfere with the measurement, it was necessary to make sure that the signals detected originated from proteins and not from interfering substances.

For method development, a bottle (750 ml) of sparkling wine was treated with 79 mg proteinase (specificity see Table 1) and incubated for 18 h at 30 °C to degrade sparkling wine proteins. Since they derived from the same sparkling wine, the amount and concentration of interfering compounds was identical in the proteinase-treated and untreated sample. The samples only differed in their protein composition due to the treatment with proteinase. All samples were filtered through a 0.2 µm filter to remove insoluble substances before measurement.

In the first approach, 20 µl of proteinase-treated and untreated white sparkling wine were directly applied on the column without any further treatment and the obtained peak spectra compared (Figure 11 A). Both showed a low and stable baseline and a peak of a very high intensity in the beginning, followed by several peaks up to an intensity of 400 mAU. Nevertheless, the spectra derived from the proteinase-treated sample did not differ from the

spectra of the untreated sparkling wine. Consequently, no protein degradation was visible suggesting that most of the peaks visible in both spectra originate from interfering substances.

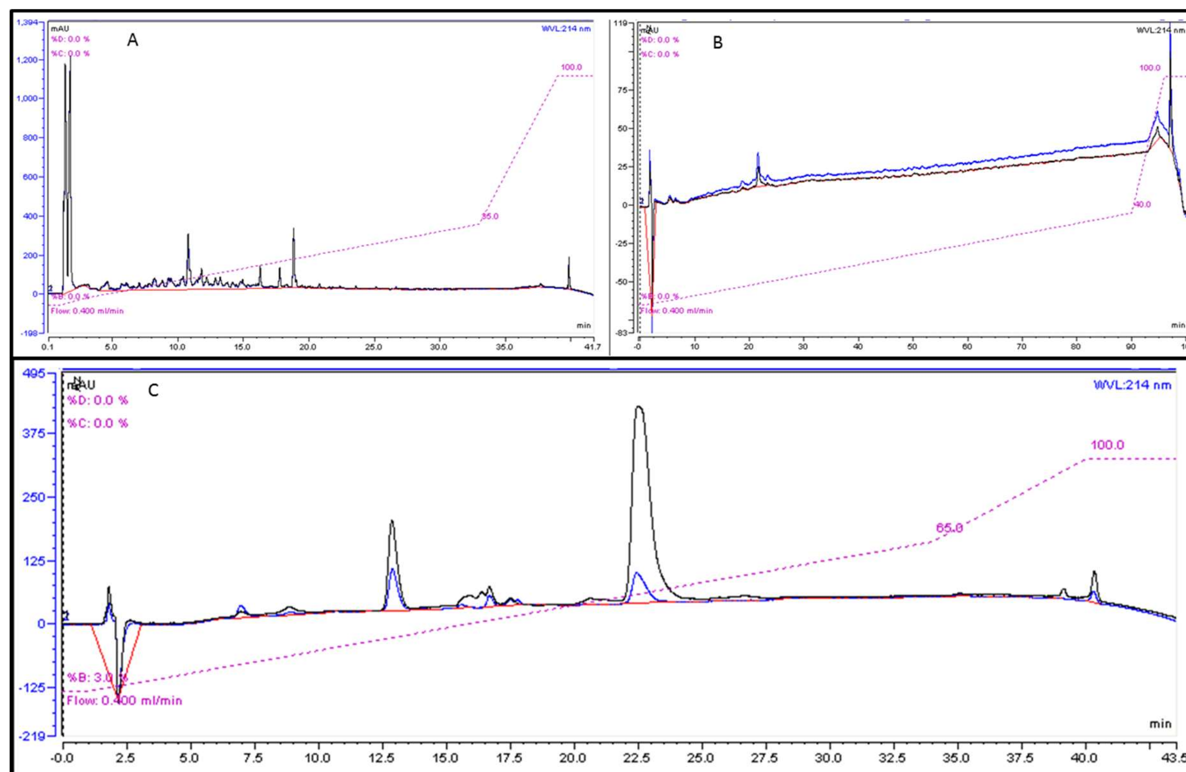


Figure 11: RP-HPLC chromatograms measured at 214 nm of white sparkling wine (black) and white sparkling wine treated with proteinase (blue); (A) untreated samples; (B) dialyzed and freeze-dried; (C) protein purification via water-saturated phenol; the dashed lane indicates the percentage of HPLC buffer B

In the next approach, the samples were dialyzed to remove low molecular weight substances, freeze-dried and dissolved in HPLC Buffer A with a 4-fold concentration. Twenty µl of the sample were applied to RP-HPLC analysis. Even if the applied concentration was 4 times higher than in the previous measurement, fewer peaks with lower intensities were visible than in the chromatogram of the unpurified sparkling wine (Figure 11B). Consequently, it was suggested that most of the peaks detected in the first approach originated from interfering substances and that they had been removed during dialysis. Nevertheless, also in this purification approach there was no visible difference between the proteinase treated and untreated samples. The dialyzed and freeze-dried samples were also applied in higher concentrations. However, no significant increase in the number of peaks and their intensity occurred. In addition, there was no visible difference between the untreated and proteinase treated samples (data not shown).

It was assumed that the low intensity of the signals is due to the low protein concentration in sparkling wines. Consequently, the proteins should be applied on the RP-HPLC in high

concentration and purity. In the following experiment, 25 ml of the sparkling wine were purified with the protocol as described in section 3.3.1 using water-saturated phenol. The proteins were dissolved in 400 µl aqueous buffer and 20 µl were applied (initial volume: 1.25 ml of sparkling wine). To achieve a complete resolution of the phenol-denatured samples in an acidic aqueous buffer such as HPLC buffer A, it was necessary to treat the protein pellet with alkaline buffers. Finally, it turned out that a successive addition of 50 µl 0.1 M NaOH followed by a stepwise addition of 50 µl 8 M Urea, 12 µl acetonitrile and 288 µl 0.1 % trifluoracetic acid in ddH$_2$O to the protein pellet resulted in chromatograms with a high resolution and reproducibility. The chromatograms showed several peaks with a maximum intensity of about 400 mAu (Figure 11 C). In contrast to the other measurements carried out previously, there was a difference visible in the chromatograms of the proteinase-treated and untreated sparkling wine. The peak areas of the proteinase-treated sparkling wine tended to be smaller than those of the untreated sample. The reduction of peak areas was due to the protein degradation caused by the enzymatic activity of the added proteinase. Consequently, the peaks monitored originated from proteins and it was assumed that the non-proteinaceous contaminants had been successfully removed by protein purification via water saturated phenol. This protocol was carried out for all quantitative measurements of grape derived beverages in this study. The exact protocol for the protein analysis via RP-HPLC is described in section 2.6.9.

3.3.3. **ELISA development for the detection of ns-LTP1 from *V. vinifera***

For the quantitative comparison of the amount of ns-LTP1 from *V. vinifera* in grape derived beverages, a suitable detection method had to be developed. Wines and sparkling wines are known to contain low concentrations of proteins. Because of its high sensitivity, a competitive ELISA was chosen as the appropriate detection method. The peptide used for the coating of microtiter plates as well as the antibody used were designed in previous studies by Specker (2014), who used them for the detection of ns-LTP1 from *H. vulgare*. The antibody is directed against the peptide used for solid phase coating. The peptide is equivalent with the amino acids of position 71 – 86 the C-terminal end of ns-LTP1 from *H. vulgare* located in a random coil structure.

In the first step the protocol was tested for its applicability using freeze dried supernatant of a recombinant *Pichia pastoris* culture overexpressing ns-LTP1 from barley that had been transformed by Specker (2014). The freeze-dried supernatant was dissolved in ELISA-PBS

buffer in different concentrations ranging from 0 – 100 mg/ml in a 100 µl volume, respectively. The result of the ELISA assay revealed that the used protocol was suitable for the detection of low concentrations of the target protein in the sample.

In Figure 12 the concentration of the freeze-dried supernatant used was plotted against the absorption at 405 nm. As expected, the less concentration of sample was used, the higher was the measured absorbance of the assay. A linear correlation between protein content and absorbance was found between 0 - 40 mg/ml of dissolved supernatant. The results revealed that the protocol applied is suitable for the peptide-antibody set designed by Specker (2014) for the detection of ns-LTP1 from *H. vulgare*.

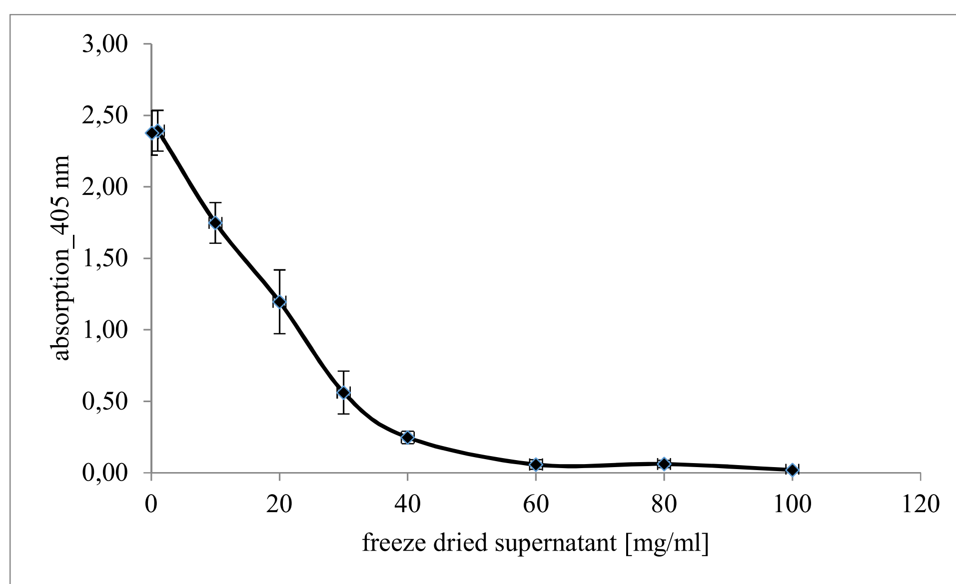


Figure 12: ELISA assay for the detection of ns-LTP1 in a freeze-dried supernatant of a *Pichia pastoris* overexpressing ns-LTP1 from *H. vulgare* dissolved in ELISA - PBS buffer. The protein concentration is plotted against the absorption at 405 nm.

Since the ELISA was supposed to be applied for the detection of ns-LTP1 from *V. vinifera,* investigations were carried out to demonstrate whether the antibody used was able to bind also ns-TLP1 from *V. vinifera*. Therefore, the amino acid sequence of the peptide that was used for plate coating and antibody design was compared with the amino acid sequence of ns-LTP1 from *V. vinifera*.

The alignment shown in Figure 13 demonstrates that peptide sequences share a 67 % homology with the protein from *V. vinifera.* The peptide consists of 16 amino acids and has 10 of them in common with ns-LTP1 from *V. vinifera* at the C terminal end. An analysis via YASPIN (details see section 2.4) for secondary structure prediction of this region revealed that this region consists of a random coil structure also in the *V. vinifera* protein. Based on

this parallels it was assumed that the peptide and antibody designed in the work of Specker (2014) should be also suitable for an ELISA assay for the detection of ns-LTP1 from *V. vinifera*. As a control, an ELISA applying different initial volumes of sparkling wine was carried out.

```
V.vinifera.ns-LTP1   MGSSGAVKLACVMVICMVVAAPAAVEAAITCGQVASSLSQCINYLQKGGAVPPGCCSGIK 60
ns-LTP1-P2           ------------------------------------------------------------

V.vinifera.ns-LTP1   SLNSAAKTTGDRQTACKCLKTFSSSISGINFGLASGLPGKCGVSVPYKISPSTDCSKVT 119
ns-LTP1-P2           --------------------------------------SKCNVNVPYTISPDID-----
                                                           .**.*.***.***. *
```

Figure 13: comparison of the amino acid sequence of ns-LTP1 from *V. vinifera* and the peptide designed for the production of polyclonal antibodies

For sample preparation, white sparkling wine was dialyzed and freeze-dried. The sample was resolved in ELISA-PBS buffer in 5 different concentrations. For the measurement, 100 µl representing an initial sample volume of 10 ml, 20 ml, 30 ml, 40 ml and 50 ml of sparkling wine were assessed, respectively. For data evaluation, the initial volumes were plotted against the absorption at 405 nm. As shown in Figure 14, the curve obtained had a similar shape than the curve obtained measuring the freeze-dried supernatant of *Pichia pastoris* overexpressing ns-LTP1 from *H. vulgare* (see Figure 12).

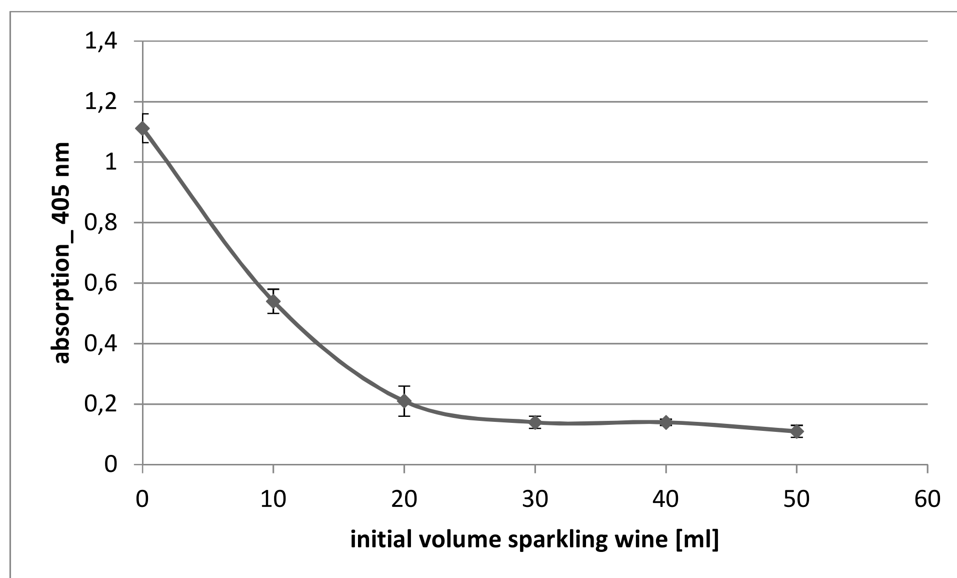


Figure 14: ELISA assay of for the detection of ns-LTP1 in dialyzed and freeze-dried sparkling wine resolved in ELISA-PBS buffer, the initial volume of sparkling wine is plotted against the absorption at 405 nm.

The less sparkling wine was assessed, the higher was the absorption signal. Since the curve obtained showed the expected progression, it was assumed that the antibody used for the

detection of ns-LTP1 form *H. vulgare* was also able to bind to ns-LTP1 form *V. vinifera* and is therefore suitable for the detection of this protein in sparkling wine. The slope of the curve had its maximum in the range of 0 – 20 ml initial volume of sparkling wine. Thus, for the following measurements, initial volumes of 5 or 10 ml sparkling wine were used. The exact protocol for the ELISA assay is described in section 2.6.10. Further protein purification as were carried out for the HPLC analysis, was not considered to be necessary in the ELISA assay. As the principle of an ELISA assay is based on protein-antibody interaction, it is more important to keep the proteins in their native state than to remove phenolic compounds.

3.4. Comparison of the protein profile of gushing and non-gushing sparkling wines

To compare the proteomic composition in gushing and non-gushing sparkling wines, a red gushing and non-gushing sparkling wine of similar make were assessed by SDS-PAGE with subsequent silver staining.

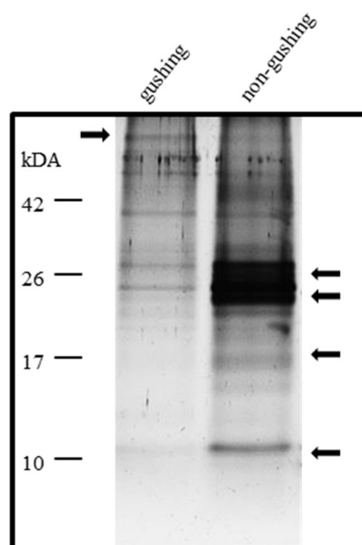


Figure 15: SDS-PAGE analysis with subsequent silver staining of a gushing and non-gushing red sparkling wine; protein bands that were analyzed via nano-ESI-LC-MS/MS are indicated by black arrows.

Results shown in Figure 15 revealed that the most prominent protein fraction in sparkling wine consisted of proteins with molecular weights between 20 kDa and 35 kDa. However, in the gushing sparkling wine, the staining of the protein bands below 35 kDa was much less intensive than in the non-gushing sparkling wine. One additional protein band occurred exclusively in the gushing sparkling wine in a molecular weight range highly above the 42 kDa marker (see arrow head on left lane in Figure 15). The protein profile of gushing

sparkling wine showed a considerable reduction in staining intensity indicating that the concentration of proteins was strongly reduced when compared to a non-gushing sparkling wine.

For the identification of those proteins which were present in non-gushing sparkling wine and absent or present in considerably reduced amounts in the gushing sample, four protein bands with a size of 13 kDA, 18 kDa, 25 kDa and 26 kDa (see arrow heads on right lane Figure 15) were excised from a SDS-PAGE gel of non-gushing sparkling wine, analyzed via nano-ESI-LC-MS/MS and subsequently identified using Mascot and the UniProt database. A protein band, noticeably larger than 42 kDa (see arrow head in left lane of Figure 15), which was exclusively present in the gushing sparkling wine, was treated in the same way.

Table 11: proteins identified in a gushing and non-gushing sparkling wine via nano-ESI-LC-MS/MS

ExpMW[1]	Th MW[2]	identified protein (organism)	accession	observation in the gushing sparkling wine	Cov[3] [%]	MS[4]	emPAI	total /unique peptides[5]
13	12.93	unnamed protein (*V. vinifera*)	CBI35210	absent	49.7	657	1.20	7 / 7
18	12.93	unnamed protein (*V. vinifera*)	CBI35210	absent	36.4	207	1.20	4 / 4
25	21.25	hyp. protein (*V. vinifera*)	CAN66515	reduced	49.3	693	1.74	28 / 6
26	21.25	hyp. protein (*V. vinifera*)	CAN66515	reduced	59.1	703	1.42	35 / 5
> 42	61,43	lcc2, laccase (*B. cinerea*)	CCD44233	exclusively present	19.4	478	0.74	13 / 13

[1]Exp MW = apparent molecular weight due to SDS-PAGE analysis;

[2]Th MW = Theoretical molecular weight, calculated based on the amino acid sequence without signal sequence;

[3]Cov = percentage sequence coverage;

[4]MS = Mascot Score; [5]total /unique peptides = count of the total peptides and the unique peptides matched to the protein (peptide sequences can be found in the supplemental material)

The protein band at 13 kDa was identified as the uncharacterized protein D7TXF5 (UniProt database entry) originating from *V. vinifera* (accession number NCBI: CBI35210.3) with a

theoretical molecular mass of 12.93 kDa. The protein band at 18 kDa was found to be the same protein. Both proteins had high sequence coverage, and a high number of unique peptides were detected in both cases (Table 11; identified peptides see appendix: Table 14). Although there is no detailed information available about protein D7TXF5, functional analysis using InterPro revealed that the protein contains the barwin domain. Proteins containing the barwin domain are in general rated to the PR-proteins of plants, as discussed later in the text (see section 4.3).
The identification of the other two protein bands with the size of 25 kDa and 26 kDa, that were both abundant in non-gushing sparkling wine revealed the same identity for both proteins. Both had a high sequence coverage and high number of unique peptides (see Table 11; identified peptides see appendix: Table 14). This protein was identified as the uncharacterized protein A5C9F1 (UniProt database entry) originating from the vine plant *V. vinifera.* (accession number NCBI: CAN66515.1) According to the analysis via InterPro this protein contained a thaumatin domain. Proteins containing the barwin or thaumatin domain are usually rated to the group of PR-proteins as mentioned in the discussion (see section 4.3).
The protein band which was exclusively present in the gushing sparkling wine with a size noticeably higher than 42 kDa was identified as a laccase from *B. cinerea* (accession number NCBI: CCD44233.1). This was an interesting result since the occurrence of a *B. cinerea* related protein in the gushing sparkling wine suggests that, it might be a result of a botrytized base wine used for its production.
In the following experiment 5 red sparkling wines were analyzed via SDS-PAGE with subsequent silver staining to compare their electrophoretic protein profile (Figure 16). In this case, no protein identification was performed. The obtained electrophoretic patterns were qualitatively compared with the patterns depicted in Figure 15. Also here, the two non-gushing sparkling wines (Figure 16 D and E) the more protein bands with a more intense staining especially in the size range between 20 kDa and 35 kDa as compared to the gushing sparkling wines (Figure 16 A and B).

A special case is depicted in Figure 16 C. This sparkling wine was classified as a gushing sample according to the manufacturer but did not show any over-foaming when opening the bottle in the laboratory. Therefore, it was assumed that the gushing potential of this sparkling wine should be lower than that of the other sparkling wines in Figure 16 A and B. Here the protein profile of the theoretical gushing sparkling wine showed more protein bands than the

other gushing sparkling wines. Summarizing the results showed that according to SDS-PAGE analysis, gushing sparkling wines tended to have a lower protein concentration and protein diversity.

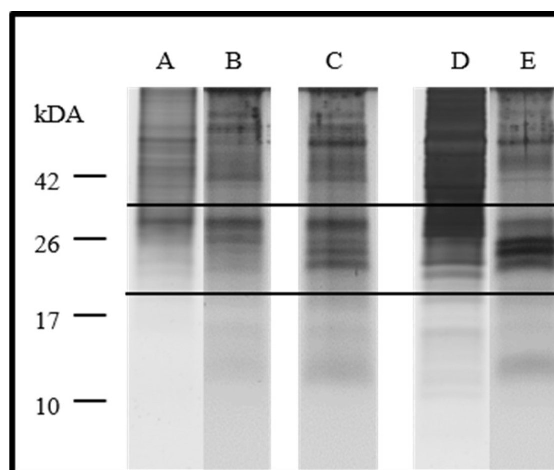


Figure 16: SDS-PAGE analysis with subsequent silver staining of (A) and (B) gushing red sparkling wines; (C) gushing red sparkling wine according to the manufacturer; (D) und (E) non-gushing red sparkling wines; protein bands between 20 kDa and 35 kDa are indicated between the black bars

However, it was not possible to draw a correlation between protein patterns and the development of gushing. In addition, it has to be considered that particles and protein haze as described in section 3.2 are potential nuclei for bubble formation and growth resulting in over-foaming. Those gushing inducing factors cannot by analyzed and detected by SDS-PAGE.

As described above, SDS-PAGE analysis turned out to be an effective tool to gain an overview about the protein profile of gushing and non-gushing sparkling wines. For a better quantitative comparison of single proteins in sparkling wines, the previously developed RP-HPLC method was applied (see section 3.3.2). In the following, protein analysis of sparkling wines will be carried out via RP-HPLC. As previously described, a protein of *B. cinerea* was identified in a gushing sparkling wine. Before further protein analysis of sparkling wines was carried out, the proteins of botrytized and healthy wines were compared to identify potential parallels between botrytized wines and the gushing phenomenon.

3.5. Influence of a *B. cinerea* infection on wine and grape proteins

3.5.1. Influence of a *B. cinerea* infection on the electrophoretic profile of grapes and wines

To gain an overview of the influence of an infection with *B. cinerea* on the electrophoretic pattern of grapes and wine proteins, respective protein extracts were subjected to SDS-PAGE with subsequent silver staining. For analysis, Pinot blanc grapes were artificially infected with *B. cinerea* in the laboratory. Grapes used for vinification originated from the same vineyard. Pinot blanc wines that were provided to the current study were made from healthy grapes and from grapes naturally infected with *B. cinerea* (see Table 8).

As shown in Figure 17 A, the protein profile of the Pinot blanc grapes after artificial *B. cinerea* infection showed some differences when compared to the profile of healthy Pinot blanc grapes, both quantitatively and qualitatively.

Results showed that proteins smaller than 35 kDa were generally less intensively stained in botrytized samples as compared to the healthy control. Moreover, some of the bands in the molecular weight range below 17 kDa were very faint or even missing in the botrytized grapes when compared to the control. Figure 17 B displays the electrophoretic pattern of the wines made from healthy and naturally infected Pinot blanc grapes. Comparison of the protein profiles after vinification with the profiles of the grapes revealed that the protein composition underwent a dramatic change during vinification.

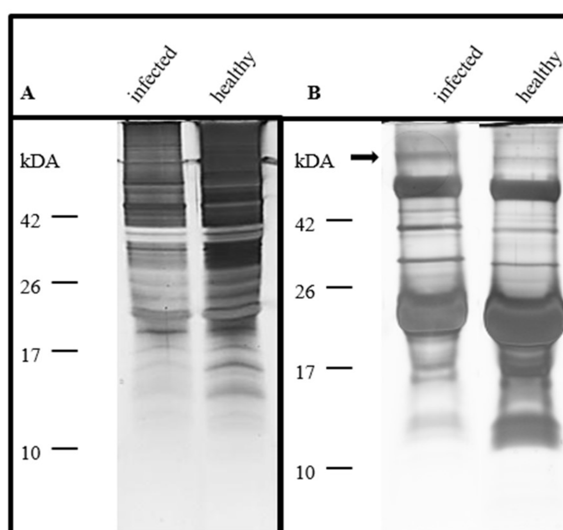


Figure 17: SDS-PAGE analysis and subsequent silver staining of (A) Pinot blanc grapes, *B. cinerea* infected and healthy; (B) wine made from B. *cinerea* infected and healthy Pinot blanc grapes made from samples described under (A).

Compared to the grapes there were less protein bands visible in the resulting wines, independently from the infection state. These results confirm earlier findings according to which vinification results in a strong decrease in protein diversity.

In both wines, the most prominent protein fraction occurred in the molecular weight range from 20 kDa to 35 kDa. Like the situation found in grapes, also the electrophoretic pattern of the wine made from infected grapes showed bands in the range from 15 kDa to 35 kDa that had either reduced intensity or were even missing when compared to the healthy control.

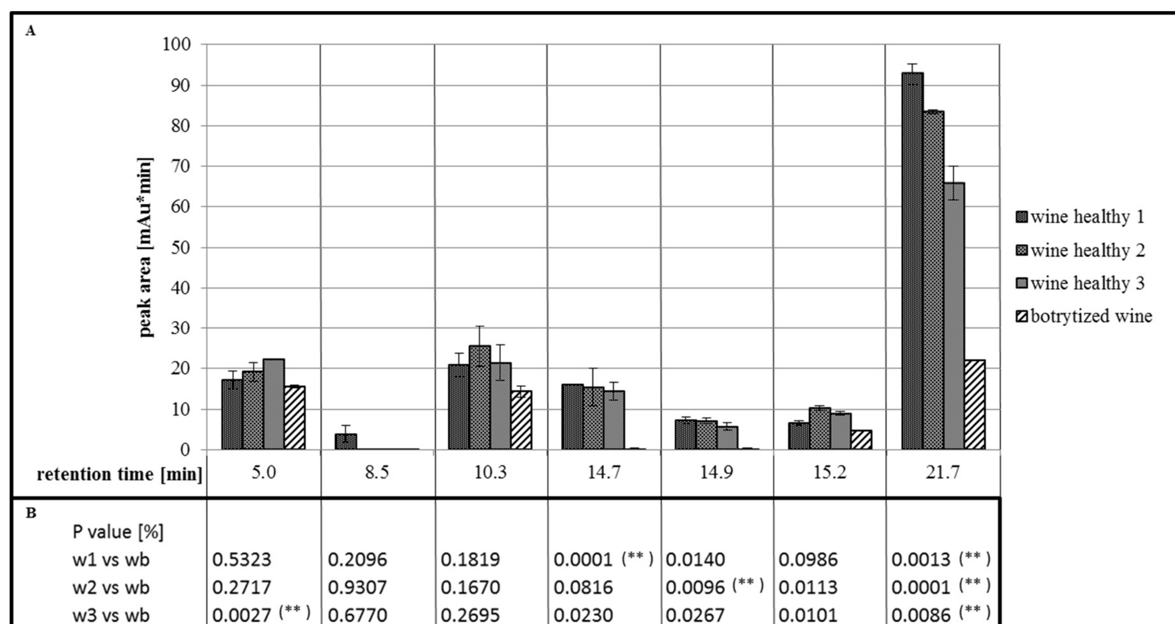
Accordingly, the changes to the protein profile induced by fungal infection were still visible in the wines even if the protein composition changed during vinification. Moreover, a protein band became more prominent in the wine made from infected grapes that had a molecular weight noticeably larger than the highest band of the used molecular weight marker (42 kDa). According to the SDS-PAGE analysis, infection with *B. cinerea* had a significant influence on the protein composition of grapes and of the resulting wine. Especially proteins in the size range of 15 kDa to 35 kDa were shown to be affected.

3.5.2. **Influence of a *B. cinerea* infection in wine visualized by RP-HPLC**

To enable a better quantitative comparison of healthy and botrytized wines, further experiments were carried out using RP-HPLC. The three Pinot blanc wines made from healthy grapes as well as the botrytized Pinot blanc wine were analyzed and the peak areas of the obtained chromatograms were compared in order to find differences caused by *B. cinerea* infection (Figure 18 A). Also, within the wines made from healthy Pinot blanc grapes, differences in the amount of the individual protein bands visible in the RP-HPLC chromatograms were observed.

Nevertheless, there was a consistent scheme visible. The wine made from botrytized grapes showed several peaks with a lower peak area and therefore reduced protein amounts compared to all three healthy controls. Statistical analysis using the T-test was conducted to identify the protein peak that is most affected by an infection with *B. cinerea* (Figure 18 B). The significance level (α) was set to 0.01. According to the T-test, there was only one protein peak, eluting at a retention time of 21.7 min, where all the p values comparing each healthy wine with the botrytized wine were below the significance level and consequently significantly different from the botrytized wine. All the other peaks did not show a consistently significant difference comparing the botrytized wines with all the three healthy wines. As described in section 0 the protein was identified as seripauperin 5 (PAU5) form

S. cerevisiae. Since this protein originates from the yeast it was not considered to be necessary to carry out a RP-HPLC analysis of healthy and botrytis grapes for the detection of the yeast protein PAU5.



P value [%]	5.0	8.5	10.3	14.7	14.9	15.2	21.7
w1 vs wb	0.5323	0.2096	0.1819	0.0001 (**)	0.0140	0.0986	0.0013 (**)
w2 vs wb	0.2717	0.9307	0.1670	0.0816	0.0096 (**)	0.0113	0.0001 (**)
w3 vs wb	0.0027 (**)	0.6770	0.2695	0.0230	0.0267	0.0101	0.0086 (**)

Figure 18: RP-HPLC analysis of three healthy wines and one wine made from botrytized grapes. (A) obtained chromatograms depicted as a bar chart. (B) comparison of the peak areas via T-test; "w" indicates wine healthy; "wb" indicates botrytized wine; ( ) indicates the p values under the significance of 0.01**

Both analytical methods, SDS-PAGE and RP-HPLC, revealed that an infection of the grapes with *B. cinerea* leads to a degradation of wine proteins. According to the RP-HPLC analysis of wines a protein eluting at a retention time of 21.7 min (PAU5) is most affected by a *B. cinerea* infection of the raw material.

3.5.3. Influence of a *B. cinerea* infection on the plant protein ns-LTP1 in grapes and wine

Hence ns-LTP1 is a grape derived protein, the influence of fungal infection can be already investigated in the grape itself. Therefore, Pino blanc grapes were artificially infected in the laboratory and a western blot analysis was conducted.

The western blot after protein purification was carried out with the same antibody used for the developed ELISA and revealed several bands with different molecular weights. The smallest band found in the size range of 10 kDa was absent in the grapes artificially infected in the laboratory (Figure 19). The most dominant band was visible at a molecular weight of about 24 kDa in the healthy grapes as well as in the infected grapes suggesting the presence

of dimers or different stages of protein glycosylation. This band was less abundant in the infected grapes. The absence or even abundance of protein bands analyzing the infected grapes revealed that an infection of the grapes with *B. cinerea* results in a lower concentration of ns-LTP1.

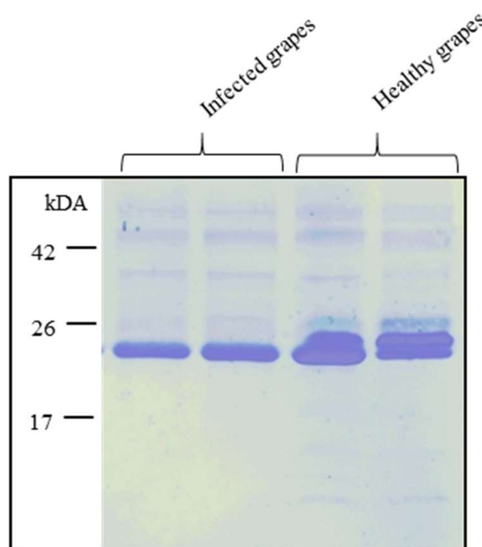


Figure 19: Western blot analysis of pinot blanc grapes infected with *B. cinerea* and healthy grapes for ns-LTP1

In addition, the three Pinot blanc wines and the botrytized Pinot blanc wine produced from naturally infected grapes that were already analyzed via RP-HPLC for their protein profile were analyzed for their amount of ns-LTP1. The ELISA assay was used to compare the concentration of ns-LTP1 in the healthy and botrytized wines. As depicted in Figure 20, all 4 analyzed samples differed in their concentrations of ns-LTP1. However, the wine made from *B. cinerea* infected grapes was the sample with the lowest amount of this protein. Even if the concentration of ns-LTP1 in the wines made from healthy grapes was not identical in all wine samples, the amount of ns-LTP1 underwent a significant reduction in the botrytized wine compared to all three samples made from healthy grapes. Compared to the healthy controls an infection of the grapes led to a degradation of ns-LTP of 70 to 77 % compared to the uninfected controls.

Summarizing the results, an infection of the grapes with *B. cinerea* has a significant impact on the plant protein ns-LTP1. Grapes artificially infected in the laboratory showed less ns-LTP1 compared to the healthy grapes. Degradation of ns-LTP1 induced by a *B. cinerea* infection is still visible in wines made from healthy and botrytized wines respectively. Wines made from infected grapes collected from the vineyard showed a reduction in the amount of ns-LTP1 of up to 77 %.

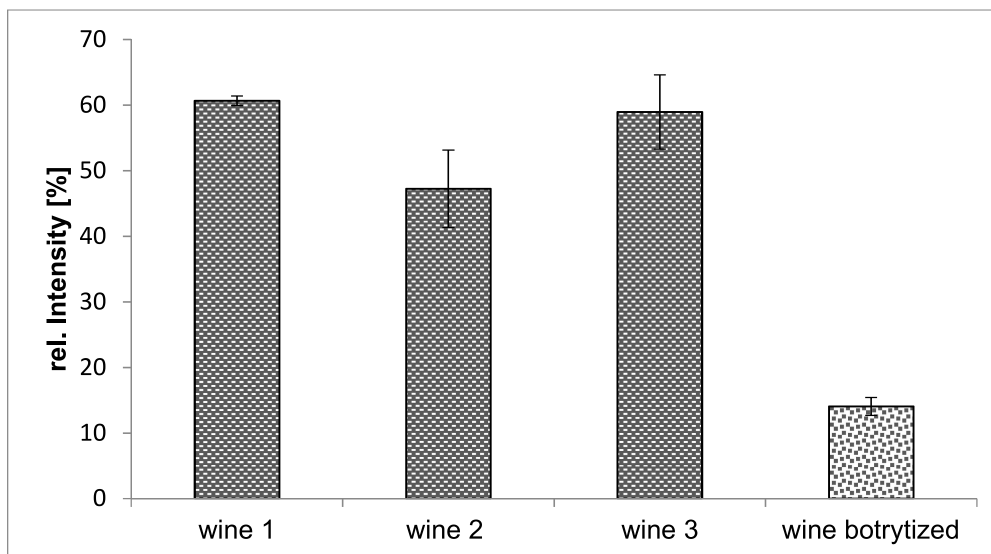


Figure 20: ELISA Assay for ns-LTP1 of three healthy pinot blanc wines (wine 1 -3) and one wine made from botrytized grapes.

3.5.4. Influence of a *B. cinerea* infection - parallels to the gushing phenomenon

According to the SDS-PAGE analysis of the healthy and infected grapes as well as the wines made from such grapes, a reduced amount of proteins was observed in infected grapes as briefly described in section 3.5.1. Especially proteins with a molecular weight lower than 35 kDa were found in significantly reduced amounts, and some were even missing in *B. cinerea* infected grapes (Figure 21 A). Also, the wines made from from *B. cinerea* infected grapes contained less protein and lower protein diversity than wines made from healthy grapes (Figure 21 B). One protein band became more abundant in the infected wine with a molecular weight noticeably larger than the highest band of the marker (42 kDa). Like the situation in grape and in wine with *B. cinerea* infection, also the protein profile of gushing sparkling wine showed a considerable reduction in staining intensity indicating that the concentration of proteins was strongly reduced when compared to a non-gushing sparkling wine (Figure 21 C).

Identification of proteins in a gushing and non-gushing sparkling wine was performed in section 3.4. Two proteins of *V. vinifera* were found to be more abundant or even exclusively present in the non-gushing sample. In addition, a laccase originating from *B. cinerea* was identified in the gushing sparkling wine. Summarizing the results, the protein profile of wine

made from *B. cinerea* infected grapes showed similar reductions and changes to the protein profile seen in the gushing sparkling wine.

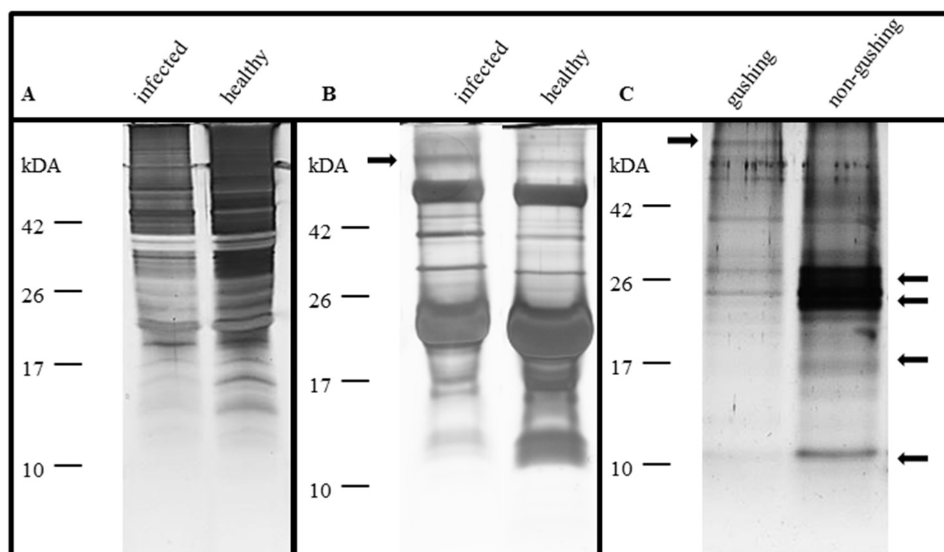


Figure 21: SDS-PAGE analysis and subsequent silver staining of (A) Pinot blanc grapes, *B. cinerea* infected and healthy; (B) wine made from *B. cinerea* infected and healthy Pinot blanc grapes made from samples described under (A); (C) red sparkling wine, gushing and non-gushing;

This was an interesting result since the occurrence of a *B. cinerea* related protein in the gushing sparkling wine suggests that, like botrytized grapes and wine, also the modification of the protein profile seen in gushing sparkling wine may be a result of a botrytized base wine used for its production.

3.6. Definition of a biomarker for gushing

3.6.1. Screening of sparkling wines for a marker protein

As described in section 3.5.4 several parallels were found between a gushing sparkling wine and a botrytized wine. To elucidate whether the observed changes in the protein composition are associated with the occurrence of gushing, a screening of sparkling wines was carried out to correlate the protein that was found to be most affected by a *B. cinerea* infection with the occurrence of gushing. The protein PAU5 form S. cerevisiae in wine was found to undergo a significant degradation in botrytized wines (see section 3.5.2).

The sparkling wines were analyzed for the presence of PAU5. They differed in grape variety, country of origin, vintage and manufacturer. To ensure that the peak found in the RP-HPLC chromatograms at a retention time of 21.7 min originated from the same protein in wines as well as in sparkling wines it was purified by RP-HPLC from a non-bortrytized wine and from

a non-gushing sparkling wine. Subsequent SDS-PAGE and MALDI-TOF MS analyses were carried out for verification of the protein (data not shown). These experiments confirmed that the protein was identical in the wine and in the sparkling wine with a protein band at 17 kDa.

Subsequently, 43 sparkling wine samples available to the study were analyzed via RP-HPLC for the presence of PAU5. A more detailed characterization of protein PAU5 will be provided in section 0 of this manuscript. To correlate the presence or absence of PAU5 with the occurrence or absence of gushing, the 43 sparkling wine samples were divided into two groups, “gushing” and “non-gushing”. However, for 8 of the samples no clear assignment to one of the two groups could be readily made because according to the manufacturer’s data the batch of each of those samples was either affected by gushing or was described to have “adverse foaming properties” occurring during the manufacturing process with no clear definition given for that attribute. No gushing or modified foaming properties were observed when those 8 samples were opened during the experiment. Those samples with unclear assignment were excluded from the screening.

Table 12: Comparison of the peak areas of PAU5 obtained by RP-HPLC of 17 different non-gushing sparkling wines and 18 different gushing sparkling wines; peak areas under the set detection limit are shaded in grey

non-gushing		gushing	
sample	**PAU5 [mAU*min]**	**sample**	**PAU5 [mAU*min]**
SW3	15.73 ± 4.12	**SW15**	291.13 ± 33.67
SW8	55.93 ± 1.96	**SWF**	90.36 ± 6.32
SW17	52.01 ± 7.09	**SWG**	44.01 ± 4.58
SW18	33.26 ± 8.10	**SWH**	21.45 ± 0.09
SW19	25.31 ± 4.01	**SWT**	47.73 ± 0.15
SW20	38.56 ± 0.61	**SWX**	49.66 ± 5.12
SW21	34.89 ± 1.93	**SWAC**	68.11 ± 5.04
SW22	124.28 ± 33.60	**SWAD**	63.49 ± 4.15
SWB	52.00 ± 1.72	**SWAE**	138.84 ± 3.44
SWAF	189.48 ± 17.37	**SW1**	≤ d.l.
SWAG	136.92 ± 8.74	**SW6**	≤ d.l.
SWAH	374.02 ± 8.41	**SW12**	≤ d.l.
SWAJ	45.86 ± 15.79	**SWE**	≤ d.l.
SWAS	586.86 ± 81.01	**SWO**	≤ d.l.
SW30	40.28 ± 2.65	**SWR**	≤ d.l.
SW31	30.64 ± 2.27	**SWS**	≤ d.l.
SWAK	≤ d.l.	**SWU**	≤ d.l.
		SWZ	≤ d.l.

After final assessment, the screening was carried out with 17 non-gushing sparkling wines and 18 gushing sparkling wines. Comparative evaluation of the RP-HPLC chromatograms revealed that 10 of the 35 sparkling wines did not contain any PAU5 or that it was present in quantities below the set limit of detection (marked as ≤ d.l. in Table 12). Nine of the samples lacking PAU5 had been assigned to the group of the gushing sparkling wines, which means that 50 % of the gushing samples did not contain PAU5 (RP-HPLC chromatograms of a non-gushing sparkling wine containing PAU5 and a gushing sparkling wine lacking PAU5 is depicted in the Appendix Figure 37).

The RP-HPLC analysis revealed that 9 of 10 sparkling wines didn´t contain PAU5 showed gushing behavior. Consequently, sparkling wines lacking PAU5 are highly susceptible to the occurrence of gushing.

3.6.2. **Characterization and identification of the marker protein**

For a more detailed characterization and identification, protein was isolated from a white non-gushing sparkling wine by RP-HPLC peak separation. Subsequently, SDS-PAGE as well as MALDI-TOF MS analysis was conducted.

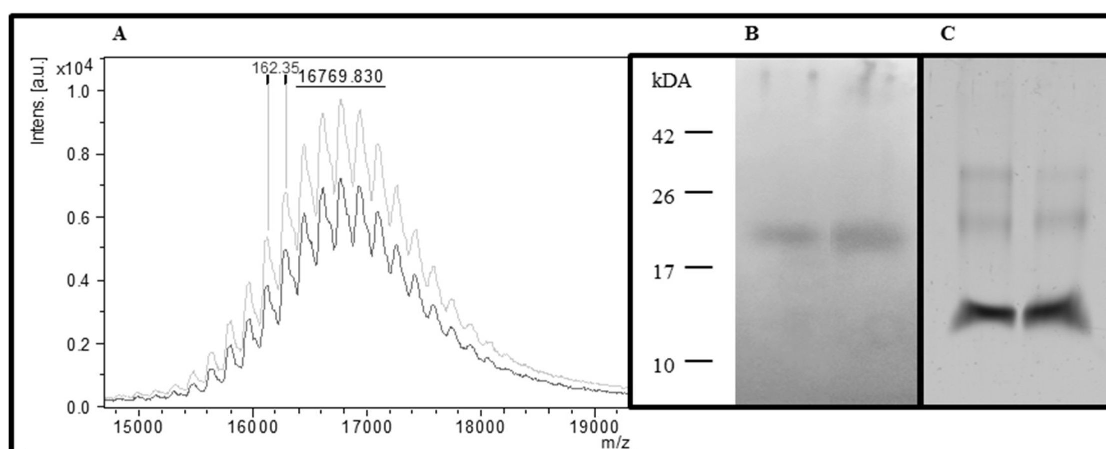


Figure 22: Analysis of protein PAU5 purified from a white non-gushing sparkling wine via (A) MALDI-TOF MS analysis, (B) SDS-PAGE analysis with subsequent silver staining; (C) SDS-PAGE analysis with subsequent silver staining after deglycosylation

The MALDI-TOF MS spectrum of the isolated protein showed a prominent peak at m/z 16,770 (Figure 22 A) and several peaks indicating a neutral loss of m/z 162, respectively, with lower intensities on both sides in a m/z range from 15000 - 18500. The data suggested that this protein is highly glycosylated. To verify the findings from the MALDI-TOF MS analysis, deglycosylation of the protein was carried out using triflouromethanesulfonic acid. The chemical deglycosylation process was preferred over enzymatic deglycosylation to avoid

interference with the deglycosylating enzyme during nano-ESI-LC-MS/MS analysis in order to gain the best possible result in protein identification. The protein was applied to SDS-PAGE before (Figure 22 B) and after deglycosylation (Figure 22 C) and subsequently silver stained. The deglycosylation process resulted in a shift of the protein band from about 17 kDa to 13 kDa, which was suggested to be due to the removal of glycans from the protein. The SDS interacts in different way with the glycan than with the amino acids resulting in a different migration behavior in the electric field than non-glycosylated proteins. In addition, the presence of glycans increase the molecular mass of the protein.

For protein identification, the band of the deglycosylated protein was excised in two independent experiments and both protein bands were sent to the Protein Analysis Unit (LMU) for nano-ESI-LC-MS/MS analysis. Matching the obtained data to the Uniprot Database identified the isolated protein as PAU5 from *S. cerevisiae* (Table 13; identified peptides see appendix: Table 14).

Table 13: Protein PAU5 identified in replicates via nano-ESI-LC-MS/MS

Sample	identified protein (organism)	accession	Cov[1] [%]	MS[2]	emPAI	total /unique peptides[3]
1	PAU5 (*S. cerevisiae*)	KZV11581	59.0	478	1.03	14 / 9
2	PAU5 (*S. cerevisiae*)	KZV11581	57.0	394	1.57	13 / 8

[1]Cov = percentage sequence coverage;

[2]MS = Mascot Score;

[3]total /unique peptides = count of the total peptides and the unique peptides matched to the protein (peptide sequences can be found in the supplemental material)

3.6.3. Isolation of the marker protein and analysis of foam stabilizing properties

Protein PAU5 was isolated as described in semi-preparative amounts via RP-HPLC from a white non-gushing sparkling wine and treated with proteinase K. To verify the success of protein purification and proteinase digestion, the untreated PAU5 protein as well as the proteinase-treated protein and the proteinase K itself were analysed by SDS-PAGE with subsequent silver staining (Figure 23).

The SDS-PAGE analysis of the purified PAU5 showed one single band with a molecular weight of approximately 17 kDa. The addition of proteinase resulted in a complete vanishing

of this band and the appearance of several protein bands with molecular weights smaller than 15 kDa. The analysis of the proteinase itself indicated a similar protein pattern to the one observed after digestion of protein PAU5 treated with proteinase. Results of the SDS-PAGE analysis showed that the purified PAU5 protein from sparkling wine was completely digested by proteinase. However, no interpretation of the results was possible regarding the size of the resulting fragments since no difference between banding patterns of the pure proteinase K and the digested PAU5 was visible.

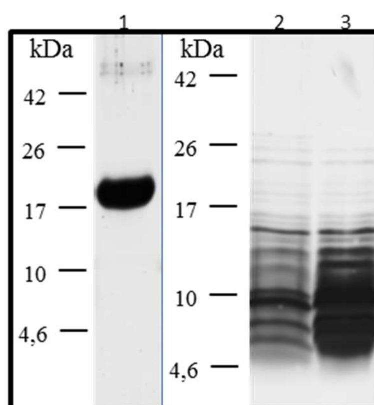


Figure 23: SDS-PAGE analysis and subsequent silver staining; (1) isolated PAU5; (2) digested PAU5 with proteinase K; (3) proteinase K

To analyze the foaming properties of PAU5 and proteinase digested PAU5, untreated and digested proteins were added to white grape juice in different amounts. The untreated grape juice displayed the most unstable foam with a foam decay time of 43.5 s. The foam stability of the grape juice treated with 40 µl (final conc. 18.67×10^{-3} mg/ml) of the purified PAU5 did not show any significant difference compared to the untreated grape juice (Figure 24). The addition of 60 µl (final conc. 28.00×10^{-3} mg/ml) PAU5 to the juice resulted in a 25 % higher foam stability compared to the untreated control. The presence of 70 µl (final conc. 32.67×10^{-3} mg/ml) of the purified PAU5 in the grape juice resulted in an increase of the foam stability of more than 170 %. The addition of the heat inactivated proteinase (final conc. 106.67×10^{-3} mg/ml) led to a slight increase in the foam stability of grape juice of approximately 20 % even though the protein concentration added was more than three times higher than the highest concentration of untreated PAU5.

Foam stabilities in samples containing the digested protein PAU5 did not affect foam stability. The presence of digested protein added did not differ significantly from juice treated with proteinase K. While the foam-stabilizing effect of PAU5 depended on the amount of the protein added to the grape juice, the digestion of this protein with proteinase K resulted in a

complete loss of its foam-stabilizing properties. Consequently, the protein PAU5 has foam stabilizing properties in the modification present in sparkling wine.

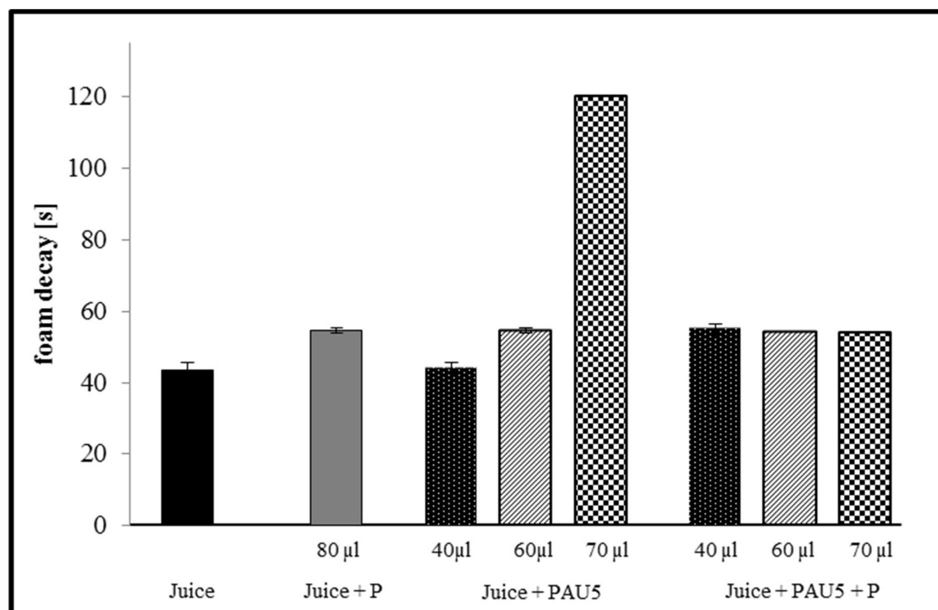


Figure 24: Analysis of the foam stability; (juice) untreated grape juice; (juice+P) grape juice with inactivated proteinase K; (Juice+PAU5) grape juice with PAU5; (Juice+PAU5+P) grape juice with the digested protein PAU5. Numbers below the columns show the volume added to 37.5 ml of grape juice.

3.6.4. **Analysis of base wines for their amount of PAU5 and foam stability**

Eight red base wines and 13 white base wines for sparkling wine production were provided for analysis by sparkling wine manufacturers. The base wines differed in vintage, grape variety, producer, country of origin and vinification process.

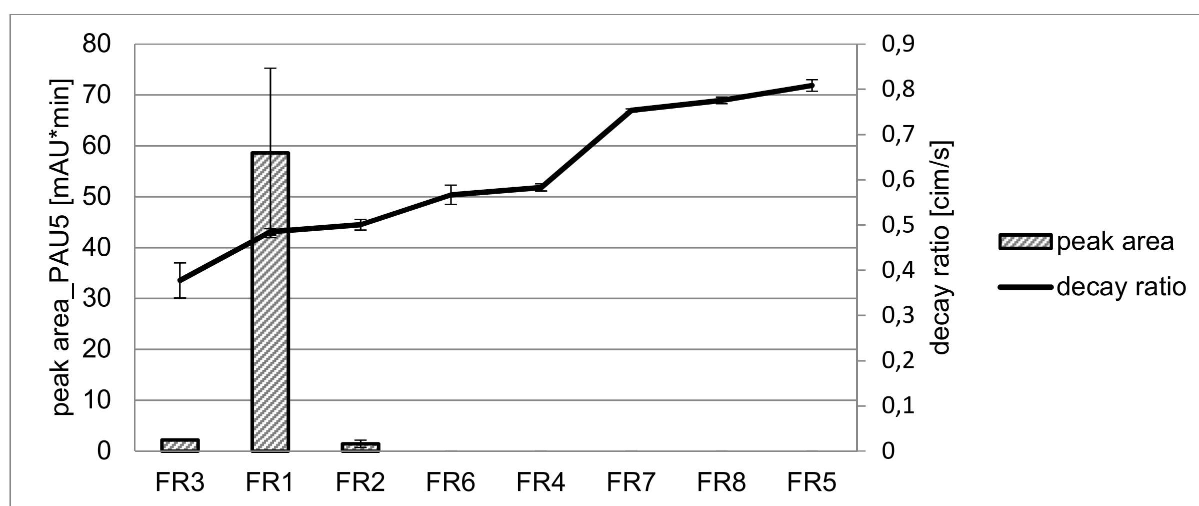


Figure 25: Bar chart representation of peak areas for the PAU5 peak in RP-HPLC analysis and with a simultaneous representation of the foam decay ratio of 8 different red base wines

The foam decay ratios determined for the red base wines were ranging between 0.377±0.039 cm/s and 0.808±0.007 cm/s (Figure 25). The RP-HPLC analysis revealed that 5 of the 8 red base wines were lacking PAU5. Interestingly, the 3 base wines containing PAU5 were found to have to the lowest foam decay rate and therefore the most stable foam. Nevertheless, the amount of PAU5 did not directly correlate with the foam decay rate. Sample FR1 was found to contain about 27 times more PAU5 than sample FR2 and FR3, but the foam decay rate of sample FR2 was comparable to that of sample FR1. The foam decay rate of samples FR3 was even lower and the therefore the foam was more stable compared to sample FR1. The foam decay ratios of the base wines lacking PAU5 were not consistent. They were ranging between 0.583±0.008 cm/s to 0.808±0.007 cm/s.

According to the RP-HPLC analysis for the concentration of PAU5, there was only 1 white base wine (sample EL3) lacking PAU5 (Figure 26). This white base wine was found to have the highest foam decay rate (0.836±0.062 cm/s) and therefore the most unstable foam of all analyzed white base wines. All other white base wines contained PAU5 in different amounts. Also, the foam decay rates differed from 0.193±0.017 cm/s to 0.539±0.129 cm/s but there was no direct correlation between foam stability and amount of PAU5 observable.

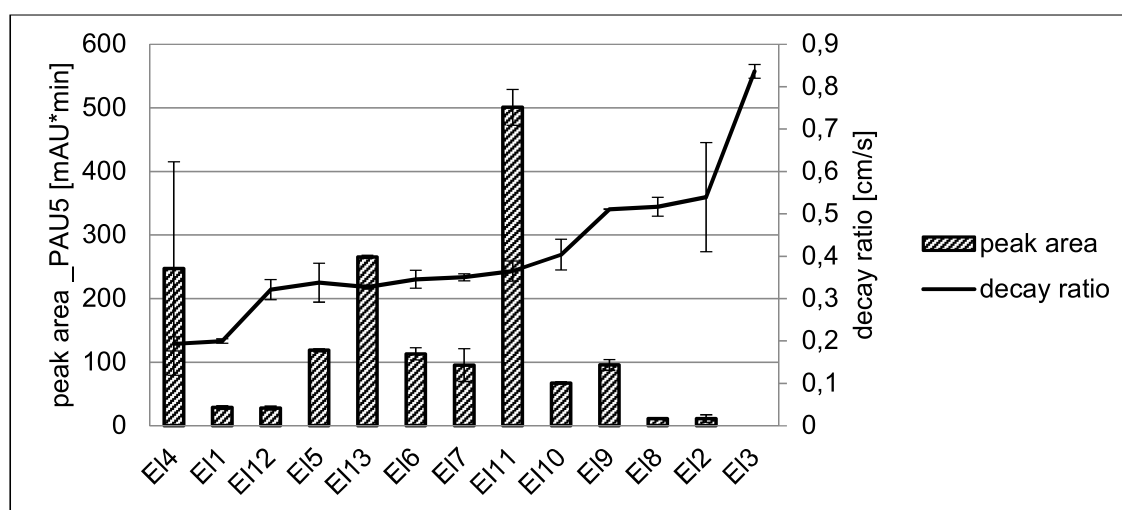


Figure 26: Bar chart representation of peak areas for the PAU5 peak in RP-HPLC analysis and with a simultaneous representation of the foam decay ratio of 13 different white base wines

Base wines lacking PAU5 were found to have the most unstable foam in the group of the red base wines as well as in the group of the white base wines. These results confirmed the findings of section 3.6.3 were protein PAU5 was found to have foam stabilizing properties. Nevertheless, it was not possible to draw a direct correlation between the amount of PAU5 in

the base wines and their foam stability. Therefore, it was suggested that also other proteins or further factors may have an influence on foam formation and stability in wines

3.7. Screening of sparkling wines for their amount of ns-LTP1

To investigate whether there is a correlation between the amount of the plant protein ns-LTP1 and the occurrence of gushing, 15 different sparkling wines were analyzed via ELISA for their amount of ns-LTP1. The sparkling wines were divided into the following three groups: non-gushing, gushing containing PAU5 and gushing lacking PAU5. The gushing samples were divided in the two groups because also the occurrence of secondary gushing was considered assuming the yeast protein PAU5 is a biomarker for primary gushing.

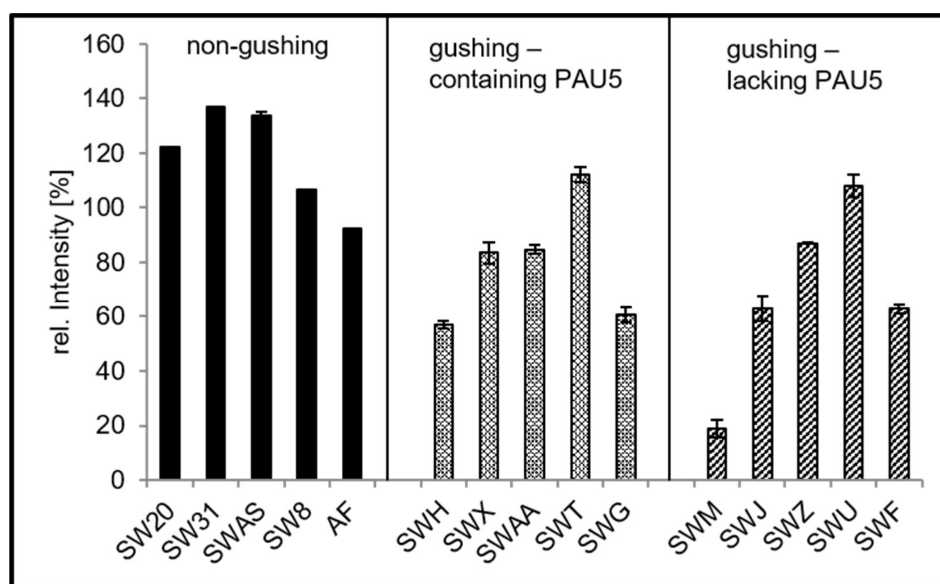


Figure 27: ELISA assay of sparkling wines for the amount of ns-LTP1; (black bars) non-gushing sparkling wines, (black-white patterned bars) gushing sparkling wines containing the marker protein PAU5, (black-white striped bars) gushing sparkling wines lacking marker protein PAU5

The ELISA assay revealed that the samples containing the highest amounts of ns-LTP1 belong to the group of non-gushing sparkling wines (Figure 27). The sparkling wine with the lowest concentration of ns-LTP1 was found to be in the group of the gushing sparkling wines lacking PAU5. Also, the gushing sparkling wines containing protein PAU5 tended to have a lower amount of ns-LTP1.

Nevertheless, it was not possible to draw a clear correlation between the concentration of ns-LTP1 and the occurrence of gushing. One of the non-gushing sparkling wines (sample AF) analyzed contained less ns-LTP1 than a gushing sparkling wine containing PAU5 (SWT) and one lacking PAU5 (SWU) respectively. Another non-gushing sample (SW8) showed a similar concentration of ns-LTP1 than the two gushing sparkling wines SWT and SWU.

Comparing the mean values as depicted in Figure 28 of the three groups of sparkling wines the non-gushing sparkling wines tended to have the highest amounts of ns-LTP1. The mean value of gushing sparkling wines containing the marker protein PAU5 showed a reduction of 33 % compared to the group of the non-gushing sparkling wines. The mean value of the gushing sparkling wines showed a reduction of even 43 %. Even if the mean values of the tree groups differ greatly from each other, no significance difference using the T-test could be determined because of the divergence in the amounts of ns-LTP1 within one of the groups.

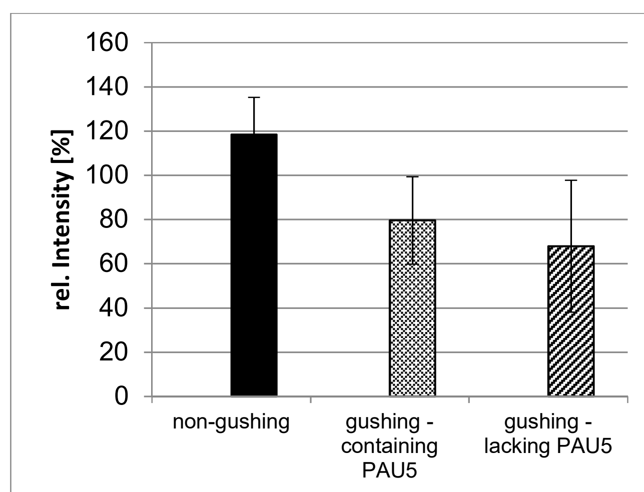


Figure 28: Mean values of the amount of ns-LTP1 in (black) non-gushing sparkling wines, (black-white patterned) gushing sparkling wines containing the marker protein PAU5, (black-white striped) gushing sparkling wines lacking marker protein PAU5

3.8. Factors influencing gushing modulating proteins and foam stability

3.8.1. Influence of the yeast strain, temperature and sulfur treatment in healthy and botrytized must

Grape juice was used as a model system for the grape must since it was yeast free and available in high amounts and produced in a standardized manner. Juice from the same production batch was used in all experiments to enable a comparison of the effect of different treatments. All samples were compared for its amounts of PAU5 and ns-LTP1, their foam stability and electrophoretic profile.

To investigate whether the presence of *B. cinerea* in grape must prior to yeast fermentation influences the amount of PAU5, ns-LTP1, electrophoretic profile and foam stability after

yeast fermentation, grape juice was inoculated with *B. cinerea* and then after a week of growth incubated with one of two different wine yeast strains (see Table 4). To investigate the influence of must treatments, grape juices were either heated or sulfurized to terminate the growth of *B. cinerea* prior to yeast inoculation.

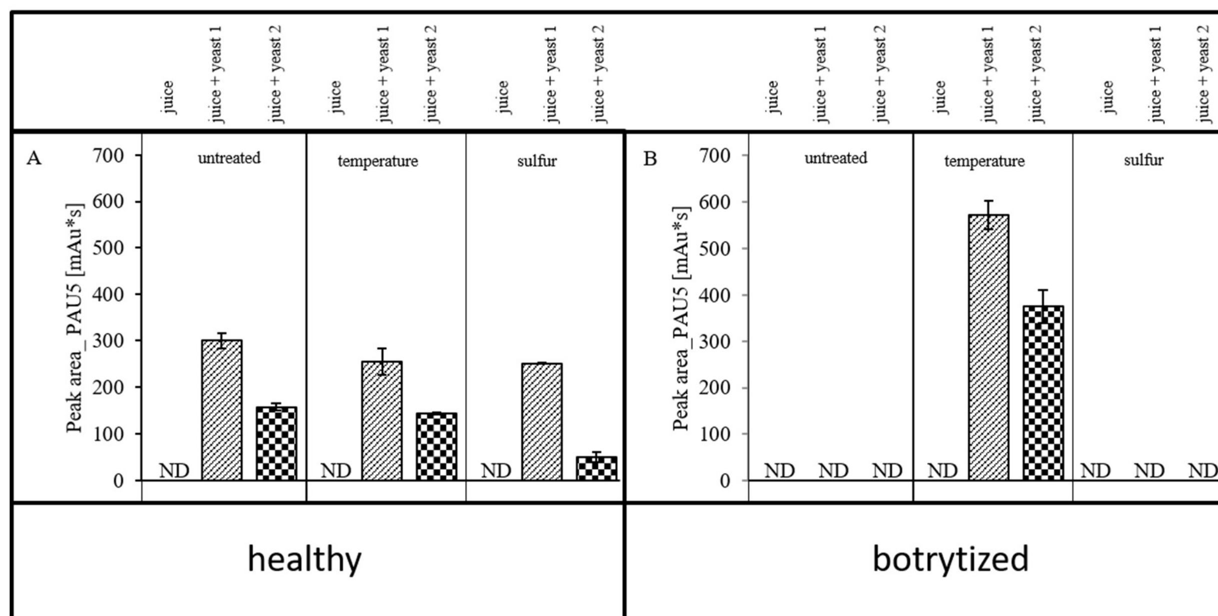


Figure 29: Bar chart representation of peak areas for PAU5 peak in RP-HPLC analysis of A) healthy grape juice untreated, with heat treatment and with sulfurization before inoculation with 2 different wine yeast strains, B) grape juice pre-inoculation with *B. cinerea* before heating and sulfurization prior to inoculation with two different wine yeast strains. (ND = not determined)

The amount of PAU5 for each sample was determined by RP-HPLC. To enable a quantitative comparison of the different samples, the mean areas of the PAU5 peaks as well as the standard deviation of the respective HPLC analysis were plotted as a bar chart (Figure 29).

No PAU5 was detectable in grape juice which had not been inoculated with yeast prior to HPLC analysis, independent from the method used for must treatment. In any case, fermentation of the *Botrytis*-free juice with yeast led to the occurrence of PAU5 with both yeast strains tested (Figure 29 A).

Higher amounts of PAU5 were observed in the samples fermented with yeast 1. Relative to this, the concentration of PAU5 produced by yeast strain 2 was approximately 47 % lower (Figure 29 A). In the presence of sulfur, the type of yeast had an even more pronounced influence since yeast 2 produced only 20 % of the amount of PAU5 found in yeast 1 after fermentation. Heat treatment of the *Botrytis*-free juice prior to yeast inoculation did not have any significant impact on the PAU5 concentration in both cases compared to the untreated juice (Figure 29 A).

No PAU5 was detectable in botrytized juice inoculated with the two yeast strains and in the botrytized samples treated with sulfur before yeast addition (Figure 29 B). The heat treatment of the *B. cinerea* infected juice before yeast inoculation resulted in even higher amounts of PAU5 compared to the healthy controls or the sulfur-treated juice (Figure 29 B).

For a comparison of ns-LTP1 in the different samples, an ELISA assay was carried out using an initial volume of 5 ml. The mean values of the relative intensities were plotted as bar charts (Figure 30). According to the ELISA assay neither heat treatment nor sulfur treatment of the juice had an influence on the amount of the protein ns-LTP1 (Figure 30 A). Consequently, this plant protein is resistant to high temperatures up to 80 °C. Also, the fermentation of the juice with one of the two yeast strains does not have an impact on the amount of ns-LTP1. An infection of the juice with *B. cinerea* lead to a degradation of ns-LTP1in the juice of about 40 % compared to the uninfected control (Figure 30 B).

The addition of sulfur after one week of infection did not prevent protein degradation in the following 10 days of further incubation. There was a significant degradation of the protein ns-LTP1 visible but no protective effect by the presence of sulfur. Heating up the juice after one week of incubation with *B. cinerea* with a following incubation for 10 days, resulted in a only 10 % decrease of ns-LTP1(Figure 30 B). Therefore, it was assumed that enzymatic protein degradation by *B. cinerea* was interrupted by heating up the samples.

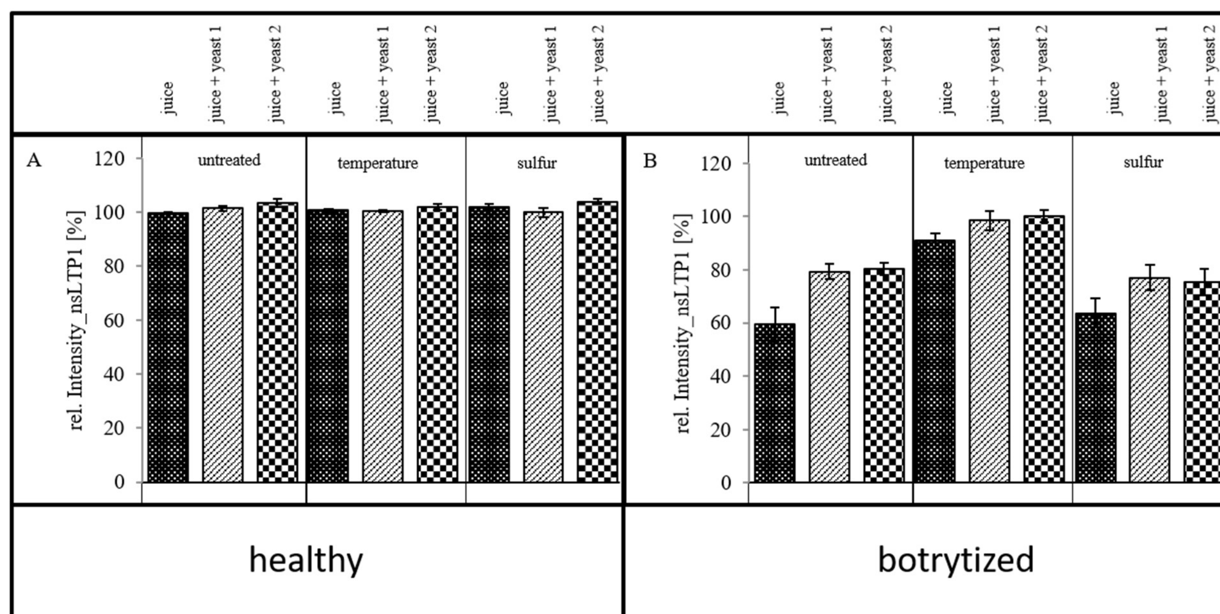


Figure 30: Bar chart representation of the relative intensity of the ELISA analysis for ns-LTP1 of A) healthy grape juice untreated, with heat treatment and with sulfurization before inoculation with 2 different wine yeast strains, B) grape juice pre-inoculation with *B. cinerea* before heating and sulfurization prior to inoculation with two different wine yeast strains.

Focusing on the proteomic patterns as depicted in Figure 31, the untreated grape juice showed a dominant band with an apparent molecular weight of approximately 25 kDa (Figure 31 A). Other protein bands were not distinguishable from each other due to poor resolution. There was no difference in the protein pattern between the untreated, sulfur- and heat-treated healthy juice without yeast addition. The fermentation of the juice with the two different yeast strains led to the appearance of additional distinct protein bands in both cases (Figure 31 A). However, there was no noteworthy difference detectable between the protein patterns resulting from different must treatments in the healthy juice (Figure 31 A). Inoculation of the juice with *B. cinerea* prior to fermentation resulted in the occurrence of additional distinct protein bands in the untreated and sulfur-treated must samples (Figure 31 B). According to SDS-PAGE analysis, there were three proteins with apparent molecular weights of approximately 35 kDa, 43 kDa, and 55 kDa that were not visible in the corresponding *Botrytis*-free samples. The proteomic pattern of the botrytized juice after heat treatment did not show any of those protein bands (Figure 31 B).

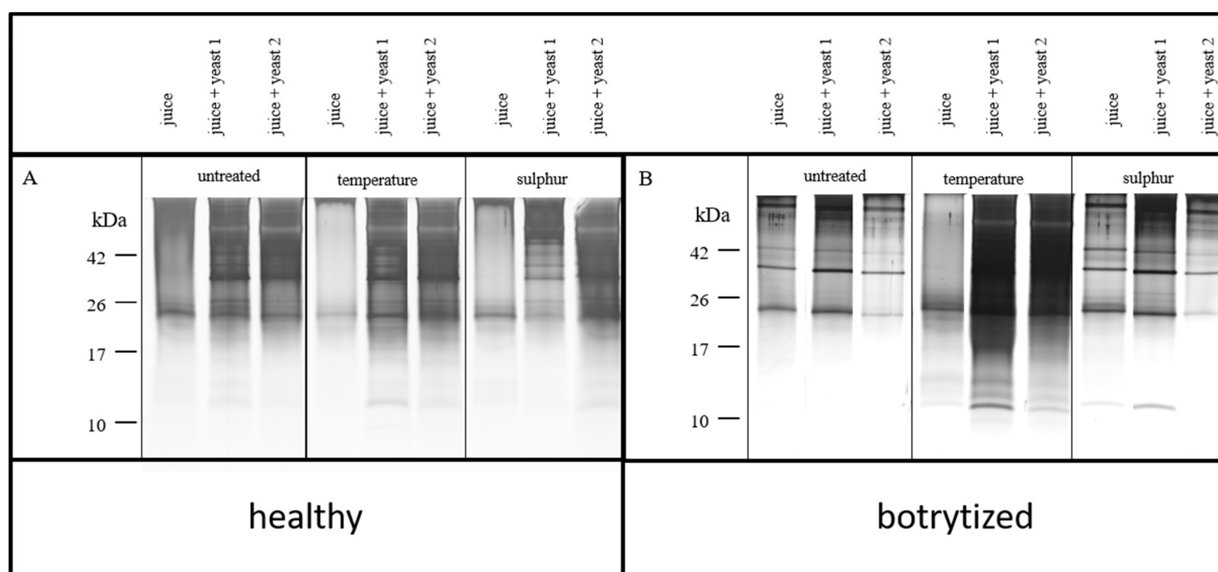


Figure 31: SDS-PAGE analysis with subsequent silver staining of A) healthy grape juice untreated, with heat treatment and with sulfurization before inoculation with 2 different wine yeast strains, B) grape juice pre-inoculation with *B. cinerea* before heating and sulfurization prior to inoculation with two different wine yeast strains.

When comparing the protein patterns of the yeast strains fermenting healthy or infected juice, there were hardly any similarities visible. Moreover, the proteomic pattern of the infected juice fermented with the yeasts had more in common with the protein pattern of botrytized but yeast free juice. The protein bands visible in the healthy juice fermented with one of the yeast strains had been completely degraded by the infection with *B. cinerea*. The addition of

sulfur in the presence of *B. cinerea* did not have a visible effect on the electrophoretic pattern of the grape juice and the grape juice fermented with yeast 1 or 2.

However, the heat treatment of the infected material with a following yeast addition resulted in a significantly different protein pattern in both cases compared to the other infected musts. The heat treatment of the infected grape juice resulted in a depletion of the protein bands found in the untreated and sulfur-treated infected juice. Especially protein bands with a molecular mass higher than 26 kDa were affected. The heat treatment of infected grape juice and a following yeast addition led to the protein profiles with the highest number of protein bands and most intensive staining in both cases and hence had the highest protein concentrations and diversity.

The foam decay of the pure grape juice, fermented with the yeasts as well as the infected samples was determined (Figure 32). The most unstable foam was found in the grape juice without any further fermentation with yeast. Neither heat treatment nor the addition of sulfur had a significant influence on the foam stability. However, the fermentation of the juice with one of the two yeast strains led in the untreated juice as well as in the heat and sulfur-treated juice to improved foam stability. The foam after fermentation with yeast was up to 3.8 times more stable than that of the pure grape juice (Figure 32 A).

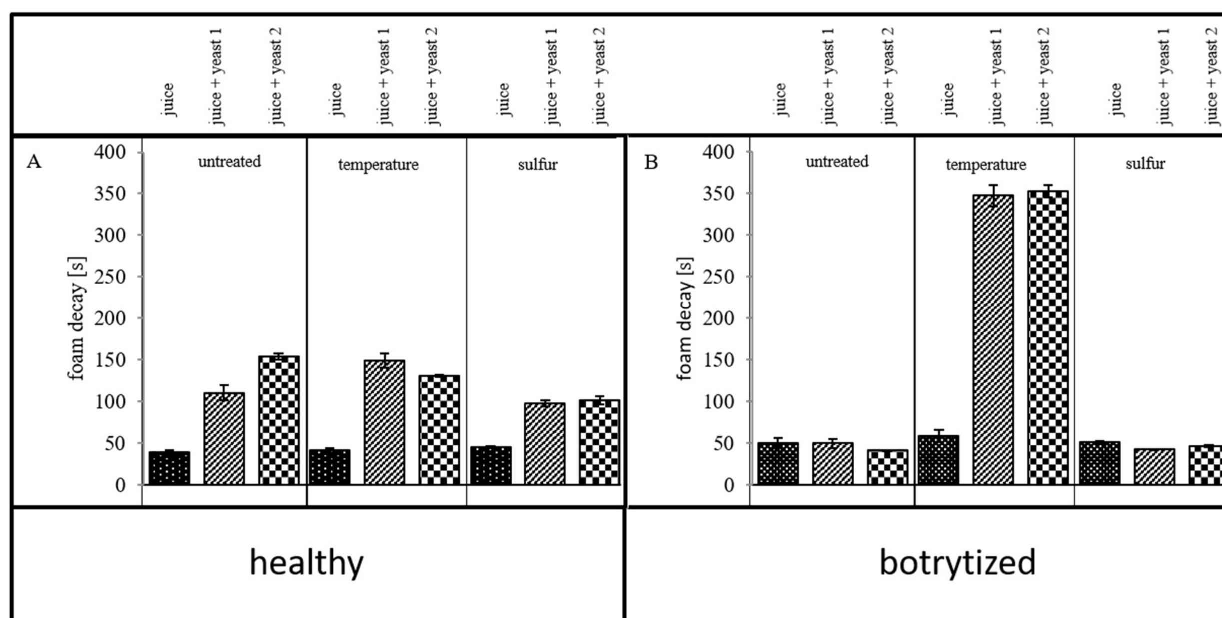


Figure 32: Measurement of the foam decay of A) healthy grape juice untreated, with heat treatment and with sulfurization before inoculation with 2 different wine yeast strains, B) grape juice pre-inoculation with *B. cinerea* before heating and sulfurization prior to inoculation with two different wine yeast strains.

To sum up results, a previous infection of grape juice with *B. cinerea* resulted in depletion of PAU5 after fermentation with any of yeasts 1 or 2. A heat treatment of *B. cinerea* infected grape juice prior to the addition of fermenting yeast showed an inhibitory effect on protein degradation and resulted in an even higher amount of PAU5 in the sample (Figure 29). The plant protein ns-LTP1 was also degraded during the pre-incubation with *B. cinerea* but not affected by heat treatment. In addition, heat treatment could stop ns-LTP1 degradation induced by *B. cinerea* (Figure 30).

SDS-PAGE analysis confirmed these finding (Figure 31). New proteins occurred after the fermentation with yeast and enhanced the foam stability of fermented juice. Those proteins that were introduced by the yeast during the fermentation process were absent when botrytized juice was used for yeast fermentation. The presence of *B. cinerea* resulted in a loss of the yeast-induced foam stability (Figure 32). In parallel, new protein bands occurred as a result of a *B. cinerea* infection. Protein degradation as well as the occurrence of new protein bands induced by a *B. cinerea* infection was prevented by heating up the samples before yeast addition. In those samples, the most stable foam was detected.

3.8.2. Influence of bentonite treatment added at different stages in the production process

To analyze to effect of bentonite-treatment on the amount of the proteins, PAU5, ns-LTP1, the electrophoretic profile and foam stability of wines, wines produced from the same batch of Portugieser grapes from the 2011 vintage were used for analysis (description of the wines see Table 7). Samples were treated with different types of bentonite added at different stages of the production process but were produced under otherwise identical conditions. All these samples as well as the untreated control were analyzed for PAU5, ns-LTP1, their electrophoretic profile and foam stability.

For quantitative comparison of PAU5 concentrations of the samples, the peak areas obtained by RP-HPLC were plotted as bar charts (Figure 33). RP-HPLC analysis revealed that the later the bentonite was added during the production process the lower was the PAU5 content of the produced wine.

Additionally, there was a consistent scheme in PAU5 removal visible. Removal of PAU5 from the wine making process was more efficient for the bentonite product from manufacturer 2 as compared to the product of manufacturer 1. Samples treated with bentonite after pre-clarification showed PAU5 concentrations very similar to those of the untreated

control. When bentonite was added to the must before inoculation with the fermenting yeast, the resulting PAU5 concentrations were even higher in the finished wine. The amount of PAU5 was approximately 45 % higher in the wine treated in the must stage with bentonite from manufacturer 1 compared to the untreated control whereas the bentonite from manufacturer 2 added at this stage of the production process increased by only 12 %. Fining of the wine before bottling removed 10 % of PAU5 compared to the control when bentonite from manufacturer 1 was used but 34 % after application of the product from manufacturer 2. PAU5 concentrations were generally lower compared to the untreated controls in the wines after wine fining even though a lower dose (250 g/hl) of bentonite was used instead of the usual 300 g/hl in the other treatments.

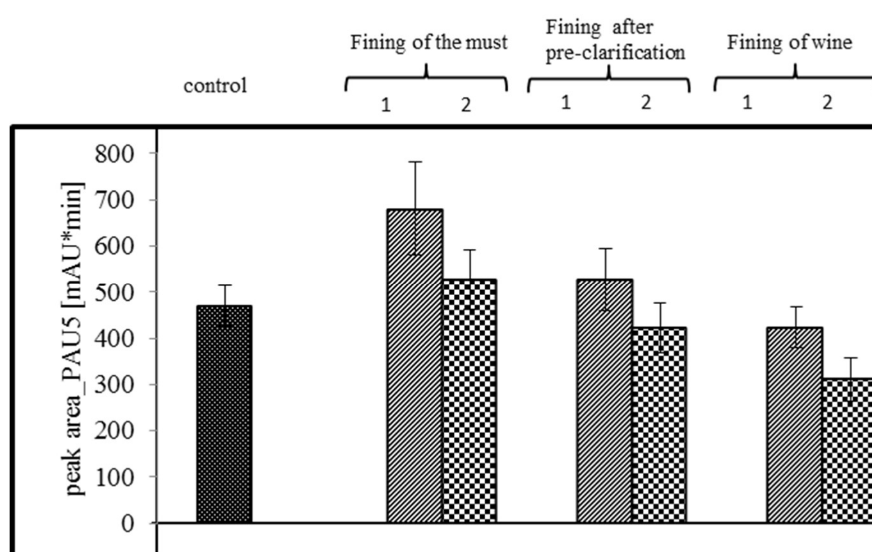


Figure 33: Bar chart representation of peak areas of the PAU5 protein peak in the RP-HPLC chromatogram of Portugieser wines treated with bentonite from (1) manufacturer 1 and (2) manufacturer 2 at different stages of the wine production process

To figure out to what extend the plant protein ns-LTP1 is affected by bentonite treatment of the wine an ELISA assay for its detection was carried out. The relative intensities were plotted as bar charts as depicted in Figure 34. Bentonite treatment of the must did not show any visible effect on the removal of ns-LTP1 using bentonite from producer 1 as well as bentonite from producer 2. Similar observations were made when the bentonite was added to the wine before bottling. Adding the bentonite in this stage of the production process, the amount of ns-LTP1 was not affected when bentonite from producer 2 was used. The addition of the bentonite from producer 1 in this last step of the production process resulted in a slightly reduced amount of ns-LTP1. However, due to the high standard deviation it was not possible to predict whether there is a significant difference in protein removal compared to

the negative control. The amount of ns-LTP1 was most affected when the bentonite was added after pre-clarification, whereby there was no significant difference between the two types of bentonite applied. The wines where the bentonite was added after pre-clarification contained 21 % and 28 % less ns-LTP1 compared to the negative control, respectively.

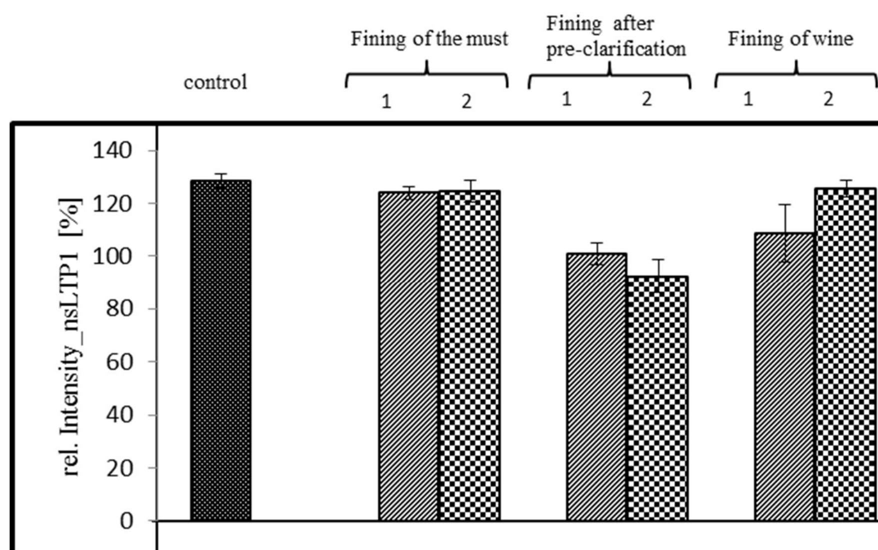


Figure 34: Bar chart representation of the relative intensity of the ELISA analysis for ns-LTP1 of Portugieser wines treated with bentonite from (1) manufacturer 1 and (2) manufacturer 2 at different stages of the wine production process

The overall electrophoretic protein pattern of the different wines was determined via SDS-PAGE analysis (Figure 35). In all samples, the most dominant protein fraction was in the range of 24 to 30 kDA. The proteomic patterns of all bentonite-treated samples were different from the untreated control. The main protein fraction affected by bentonite treatment had a molecular weight smaller than 24 kDa. The concentration of proteins in this fraction was moderately to strongly decreased compared to the untreated control.

The most protein bands and the greatest intensity of the bands were visible in the untreated control wine, followed by the samples where the bentonite was added to the must.

Bentonite treatment after pre-clarification resulted in a wine with the lowest amounts of protein bands compared to the other wines. A similar protein pattern was observed when wine fining before bottling was conducted. However, the intensity of the protein bands was higher, showing that higher concentrations of protein were present than in the wine where the bentonite was added after pre-clarification. SDS-PAGE analysis showed that there was no visible difference in the overall protein pattern using the different bentonite products.

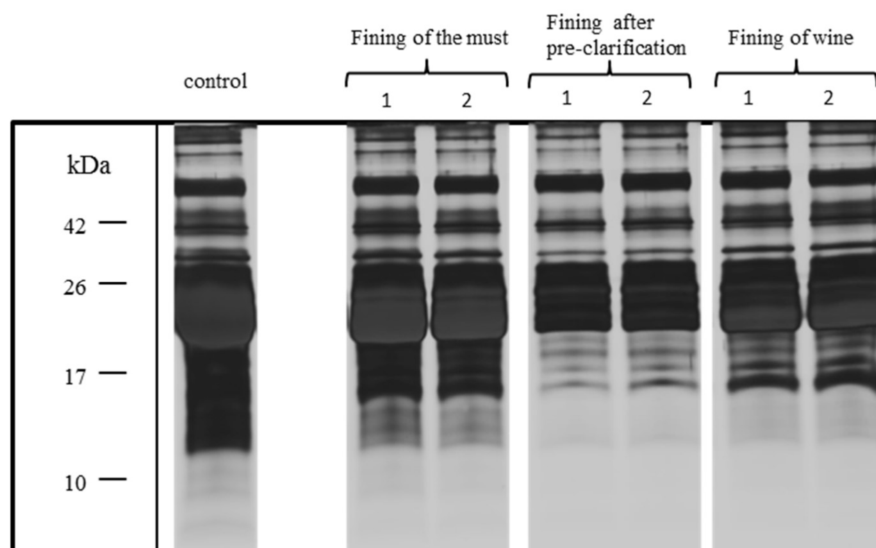


Figure 35: SDS-PAGE analysis with subsequent silver staining of Portugieser wines treated with bentonite from (1) manufacturer 1 and (2) manufacturer 2 at different stages of the wine production process

The foam stability of each wine was measured, respectively and the time of foam decay was plotted as bar charts (Figure 36). In general, there was no consistent scheme on foam decay observed. Bentonite treatment of producer 1 of the must resulted in the wine with the most stable foam. According to the preceded RP-HPLC analysis this sample contained the highest concentration of PAU5 compared to the other wines (Figure 33) whereas the amount of ns-LTP1 according to the ELISA assay did not differ from the untreated control.

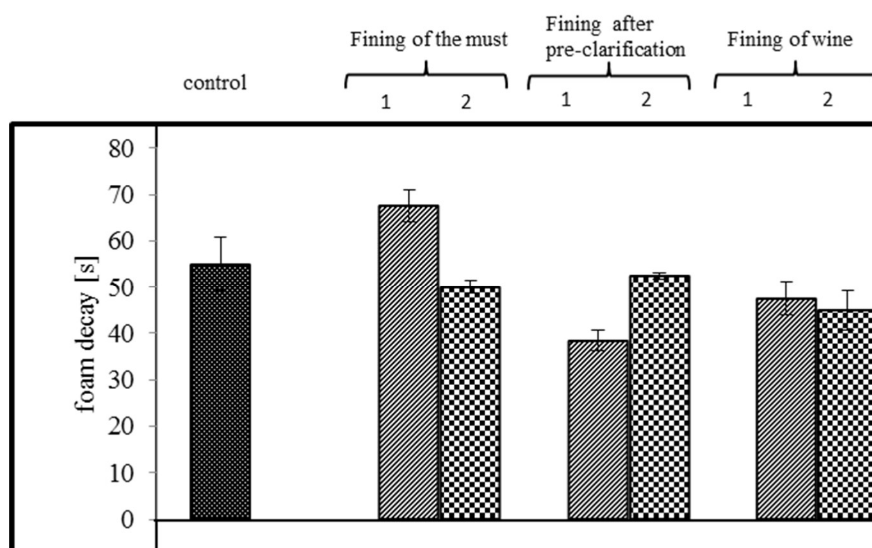


Figure 36: Measurement of the foam decay of Portugieser wines treated with bentonite from (1) manufacturer 1 and (2) manufacturer 2 at different stages of the wine production process

The poorest foam stability was found in the wine treated with the bentonite from producer 1 after the pre-clarification process. According to SDS-PAGE analysis most of the protein bands were diminished when the bentonite was added after pre-clarification. The concentration of ns-LTP1 was also most affected when the bentonite was added in that particular stage of the production process (Figure 34). According the RP-HPLC analysis the amount of PAU5 was not affected by bentonite treatment in this wine (Figure 33).

Results showed that application of bentonite during wine processing resulted in a general removal of proteins from the wine (Figure 35). However, the protein content of wines was most affected when the bentonite was added after pre-clarification. Removal of the protein ns-LTP1 followed this scheme (Figure 34). The lowest amounts of ns-LTP1 were found in the wines treated with bentonite after the pre-clarification. Even if there were differences observed in the ns-LTP1 removal by adding the bentonite at different stages in the production process, the amount of ns-LTP1 was not affected by using the different types of bentonite applied at the same production stage during vinification. The PAU5 concentration was most affected when adding the bentonite to the finished wine (Figure 33). In addition, the amount of protein PAU5 that will be removed from the finished wine turned out to depend on the type of bentonite applied. However, the removal of PAU5 by bentonite application did not correlate with the removal of the total proteins from the analyzed wines. The highest concentration of PAU5 and highest foam stability was observed after must treatment using bentonite from producer 1. In contrast, bentonite of producer 1 added after the pre-clarification led to the most unstable foam (Figure 36). In parallel, the bentonite treatment in this stage of the production process resulted in the lowest concentration of ns-LTP1 and lowest protein concentration and diversity according to SDS-PAGE analysis.

4. DISCUSSION

By the results of the current study the initial objectives resulting from the working hypothesis were achieved as described in the following:

Identification of particles that may cause secondary gushing

- Crystals, particles and protein haze were detected.

Development and optimization of protein purification and analysis strategies in wine and sparkling wine

- Development of a protein purification strategy by the use of water saturated phenol for SDS-PAGE analysis and RP-HPLC of grapes and grape derived beverages.
- Development of an ELISA assay for the detection of ns-LTP1 in grapes and grape derived beverages.

Protein chemical comparison of a gushing and non-gushing sparkling wine

- Gushing sparkling wines tend to have a lower protein amount and diversity. However, it was not possible to draw a direct correlation between the electrophoretic pattern of sparkling wines and the occurrence of gushing.
- In one gushing sparkling wine a protein from *B. cinerea* was identified, that lead to the suggestion that a botrytized base wine was used for production.
- A closer investigation of botrytized and healthy wines revealed that the protein PAU5 from *S. cerevisae* is most affected by an *B. cinerea* infection.

Identification of proteins that correlate with the absence or presence of gushing with a further characterization of those proteins regarding to their foaming properties

- A screening of sparkling wines for the presence of PAU5 revealed that the absence of this protein in sparkling wines is highly connected with the occurrence of gushing.
- PAU5 is highly glycosylated and has structural similarities to mannoproteins.
- PAU5 has foam stabilizing properties. Therefore, it was assumed that this protein has a preventing influence on the gushing potential of sparkling wines.

Identification of proteins in sparkling wine that are known to be gushing influencing from the gushing research done in beer

- Reduced amounts of the plant protein ns-LTP1 were found in gushing sparkling wines.

- It was not possible to draw a direct correlation between the occurrence of gushing and the amount of ns-LTP1 in sparkling wines.

Investigation of the influence of process parameters on the concentration of those proteins

- Infection of grapes with *B. cinerea* has a negative impact on the ns-LTP1 and PAU5 concentration in must and wine.
- Heat-treatment of infected material prevents Botrytis-induced protein degradation and resulted in even higher amounts of PAU5.
- Bentonite-treatment influences the amount of ns-LTP1 and PAU5 in the finished wine.

In the subsequent chapters of the discussion these results are supported and explained in detail.

4.1. Secondary gushing caused by crystals, residual filter aids and protein haze

The sparkling wines analyzed showed different over-foaming behavior. Over-foaming with a slow release of big bubbles over a long period of time was termed Type I whereas Type II over-foaming was characterized by a massive and quick over-foaming immediately after pressure release. However, it was not possible to clearly categorize all samples into Type I or Type II over-foaming, since the foaming behavior was also greatly depending on the temperature of the sample. The different types of over-foaming were rather an indicator that the bubble formations after pressure release by opening of the bottle were based on different types of CO_2 nucleation sites. A closer examination of sparkling wines with Type I over-foaming showed that in most of the cases the bubbles raised either from the bottom or the wall of the bottle whereas it was suggested that Type II over-foaming is caused by bubble formation all over the volume of the liquid.

In some gushing samples with Type I over-foaming, particles such as crystals and residual filter aids were detected. In this work the residual filter aids were found in a gushing carbonated grape juice. As mention in section 1.1, filtration is a common tool in wine and sparkling wine production. Therefore, it was assumed that residual filter aids may also occur in sparkling wines resulting in gushing.

As already known from the research done on beer gushing, calcium oxalate crystals as well as filter aids can act as gushing inducing factors (Zarnkow & Back, 2001; Zepf & Geiger, 2000). Therefore, it was suggested that those particles found in the gushing samples are nucleation sites resulting in excessive over-foaming. According to the classification of Gjertsen et al. (1963) this type of gushing can be attributed as secondary gushing.

In the current study, the crystals found in sparkling wines were not further characterized. According to the literature, crystals in wine usually consist either of calcium muconate or calcium tartrate (Kielhöfer & Würdig, 1961; Schanderl, 1964). Those crystals may act as nuclei for bubble growth (heterogenous “de novo nuclei”) and formation resulting in unwanted over-foaming according to the mechanism described in beer. In addition, air inclusions attached at those crystals may result in a homogenous “de novo nuclei” formation as described by (Liger-Belair et al., 2013) .

Not all crystals containing sparkling wines showed gushing behavior. Indeed, previous studies revealed that a heterogeneous bubble formation and growth depends on various

factors, such like pH, contact angle, surface hydrophobicity, surface tension and surface texture (Bankoff, 1958; Schulze, 1985; Schwuger & Findenegg, 1996; Zuidberg, 1997). As this thesis is focusing on yeast and grape derived proteins, these factors were not investigated in more detail.

One of the gushing sparkling wine samples showed altered light scattering behavior compared to the non-gushing negative control of comparable make. A closer examination revealed that aggregated proteins with a molecular weight smaller than 26 kDa was responsible for the modified light scattering properties. Protein haze or even non-visible coagulates were already discussed to contribute to gushing in beer (Bach, 2001; Curtis & Martindale, 1961). In addition, Christian et al. (2010) found putative gushing-inducing particles with sizes of 1-2 nm in apple spritzer. The presence of those particles resulted in significantly higher light scatter intensities compared to the non-gushing control.

4.2. Applicability of the developed protein purification and analysis strategies from grape derived beverages and grapes

As described in section 3.3, a new method for protein purification of sparkling wines was developed during the current work. The low protein concentration and high amount of interfering compounds make protein analysis, especially in red (sparkling) wine challenging. The developed method allows a concentration of the proteins with a simultaneous reduction of interfering compounds in juice, wine and sparkling wine as well as the grapes itself.

So far, only two studies are known in which SDS-PAGE analysis was performed with red wine (D'Amato et al., 2010; Wigand et al., 2009). In both studies, samples of red wine were treated with PVP or PVPP either directly or after freeze drying to remove the interfering phenolic substances. However, our studies revealed that sample treatment with PVP and PVPP results in a significant loss of protein concentration that did not allow proper qualitative and quantitative analysis. The new protein purification strategy developed here allows the use of SDS-PAGE with a reduced background resulting in clearly resolved bands. Although the protocol is time consuming, the improvement of SDS-PAGE resolution is considerable. Therefore, this method was applied for protein purification before SDS-PAGE analysis throughout the current study.

For a quantitative comparison of proteins, RP-HPLC was applied. RP-HPLC analysis for wine was already applied by Peng et al. (1997). However, this group used the technique for protein isolation from a white wine, but no comparison of different wines was performed. In

the current work proteinase was used for method development to ensure that the chromatograms obtained are not interfered by non-protein compounds. According to these experiments, a prior purification and concentration using the new method for protein purification with water saturated phenol was necessary to obtain high quality chromatograms.

The ELISA assay was applied to compare the amount of the plant protein ns-LTP1 in the samples. The samples were dialyzed and subsequently frieze-dried before analysis to achieve a higher concentration, but not further purified. The antibody used was directed against ns-LTP1 form *H. vulgare*. It turned out to be suitable also for the detection of ns-LTP1 form *V. vinifera.* This was expected since both proteins show a high sequence homology. Nevertheless, the sensitivity of the assay could be improved in future studies by using a more specific antibody. To the best of our knowledge this is the first time that an ELISA assay for the detection of ns-LTP1 from *V. vinifera* was developed and applied to the analysis of grape juice/grape must, wine and sparkling wine.

4.3. The parallels between the gushing phenomenon and botrytized wines and grapes

A comparison of gushing and non-gushing sparkling wines during the current study revealed significant differences in their protein composition. Like the analyzed wine, proteins in the size range from 15 kDa to 35 kDa also constitute the main group of proteins in the non-gushing sparkling wine. Protein D7TXF5 originating from *V. vinifera* (accession number NCBI: CBI35210.3) with a molecular weight of 12.93 kDa containing a barwin domain was found to be absent in the gushing sparkling wine. Two protein bands, which were well separated after SDS-PAGE analysis, at 13 kDa and 18 kDa were found to contain this protein according to Mascot analysis.

The presence of two bands of one protein in SDS-PAGE analysis can be explained by modifications such as glycosylation resulting in a shift of the protein bands. According to Dambrouck et al. (2003) glycoproteins are usually present in wines.

Another protein which was less abundant in the gushing sparkling wine was identified as protein A5C9F1 (accession number NCBI: CAN66515.1) from *V. vinifera* with a molecular weight of 21.25 kDa containing a thaumatin domain according to Mascot analysis. Also in this case a double band was visible after SDS-PAGE analysis with protein bands at 25 kDa and 26 kDa, respectively. Proteins containing the barwin or thaumatin domain are involved in

the response of the *V. vinifera* plant to bacterial or fungal attack and have thus been assumed to be PR-proteins (Ruiz-Medrano et al., 1992; Svensson et al., 1992).

A comparison of healthy and B. cinerea infected grapes revealed that, especially proteins with a molecular weight lower than 35 kDa were found in significantly reduced amounts, and some were even missing in *B. cinerea* infected grapes.

The wines made from healthy and from *B. cinerea* infected grapes contained less protein and lower protein diversity than the electrophoretic pattern of the corresponding grapes. As reported by Murphey et al. (1989) the loss of proteins during vinification is mainly related to proteolysis caused by changes in the pH value.

The changes in the electrophoretic pattern caused by the infection of *B. cinerea* were still visible in the wine. The most prominent protein fraction in the healthy wine ranges between 20 kDa and 35 kDa. The infection of the grapes causes a reduction of proteins in the resulting wine. Especially proteins smaller than 35 kDa are affected. Protein identification in the wines was not considered to be necessary and therefore not performed during the current study because other research groups already studied and identified proteins in wines to a greater extend. According to the literature most proteins within the range of 17 - 25 kDa were found to be thaumatin-like proteins. Proteins in the ranges between 26 - 32 kDa and 35 - 42 kDa, respectively, have been assigned to chitinases and β -glucanases (Esteruelas et al., 2009; Le Bourse et al., 2011; Marangon et al., 2009; Peng et al., 1997; Pocock et al., 2000; Sauvage et al., 2010). Those three classes of plant proteins are members of the family of PR-proteins (Stintzi et al., 1993; Waters et al., 1996).

The influence of a *B. cinerea* infection on the amount of the plant protein ns-LTP1 was investigated in grapes as well as in wines. Several bands were detected in the western blot analysis. The most prominent band visible in the size range of 24 kDa was less abundant in the infected grapes. The healthy grapes showed a very faint band at about 10 kDa that was even absent in the infected samples. Ns-LTP1 from *V. vinifera* has a molecular weight of 11.7 kDa. The presence of several bands with different molecular weights was suggested to be due to the presence of dimers or different stages of glycosylation that resulting in a shift of the apparent molecular mass of a protein. RP-HPLC analysis revealed that the vinification of botrytized grapes led to lower amounts of the PR-protein ns-LTP1 in the resulting wine. So far, a degradation of ns-LTP1 from *V. vinifera* in grapes, wine and sparkling wine due to fungal proteases was not described in the literature. However there are similarities to the

research carried out in beer proteins. Hippeli and Hecht (2009) stated a degradation of ns-LTP1 from *H. vulgare* by fungal proteases secreted by *Fusarium* spp. into the beer.

Comparing these findings with our observations made in the botrytized wine, it was concluded that an infection of the grapes results in a considerable degradation of PR-proteins, especially the thaumatin-like proteins, the chitinases, the β-glucanases and ns-LTP1, in the grape itself and in the resulting wine. This conclusion is in agreement with the observations made by Girbau et al. (2004) who detected reduced amounts of PR-proteins, especially the thaumatin-like proteins and chitinases in berries infected with *B. cinerea* as well as in the juice made thereof. Similar results were obtained by Cilindre et al. (2008) in Champagne base wines. The degradation of grape proteins in botrytized wine is caused by the proteolytic activity of this fungus as reported previously by Marchal et al. (2006) and Ten Have et al. (2004).

The results showed that there is a reduced amount of PR-proteins present in gushing sparkling wine compared to the analyzed non-gushing sparkling wine. According to these findings, there are parallels with the analyzed healthy and infected wines, since an infection of the grapes led to reduced amounts of PR-proteins as mentioned above.

A laccase originating from *B. cinerea* was identified in the gushing sparkling wine. This finding relates the general depletion of proteins in gushing sparkling wine to the presence of *B. cinerea* in the grapes used for making the base wine to produce the gushing sparkling wine. These findings are in agreement with the findings of Marchal et al. (2001). They found gushing behavior in Champagne made from botrytized Chardonnay grapes (40 % infection rate).

4.4. The significant decrease of the protein PAU5 from S. cerevisiae in botrytized wines

The highly glycosylated PAU5 protein from *S. cerevisiae* was found to be most affected in wines by a *B. cinerea* infection of the raw material. The concentration of this protein was significantly lower in the wines made from infected grapes as compared to the healthy wine. Therefore, it was assumed that an infection of *B. cinerea* has an impact on the amount of proteins not only originating from *V. vinifera* but also on proteins released by the yeast during the fermentation process. Degradation of these proteins can be attributed to the secretion of a group of aspartic proteases by *B. cinerea*. These enzymes are active at low pH levels and

probably remain active in an acidic environment, such as wine (Marchal et al., 2006; Ten Have et al., 2004).

PAU5 is a member of the seripauperins, the largest multigene family in *S. cerevisiae.* It is produced under low temperature and anaerobic conditions, which are predominant in the wine making process. Members of the seripauperin protein family consist of proteins 120–124 amino acids in length (Goffeau et al., 1996; Lai et al., 2005; Luo & van Vuuren, 2009; Luo & Van Vuuren, 2008; Rachidi et al., 2000; Rossignol et al., 2003). In general, seripauperins share a homology of approximately 90 amino acid residues with the N-terminal region of Tir/Dan proteins. The seripauperins were predicted to be secreted into the culture medium whereas the Tir/Dan proteins were characterized as cell wall mannoproteins containing a glycosyl-phosphatidyl inositol moiety to anchor them in the cytoplasm membrane (Abramova et al., 2001; Viswanathan et al., 1994).

Although the exact function of these proteins is not fully understood, it has been established the protein is mannosylated in its soluble form which is accompanied by enhanced stability (Luo & Van Vuuren, 2008). The MALDI-TOF-MS analysis carried out in our study revealed that the PAU5 protein present in wine and sparkling wine is highly glycosylated. Analysis of spectra revealed several isoforms differing by 162 m/z units corresponding to the molecular weight of hexose, respectively. So far, mannoproteins are the only proteins from *S. cerevisiae* known as proteins with positive influence on the foaming properties in sparkling wine (Núñez et al., 2006).

4.5. PAU5 as a biomarker for gushing in sparkling wines

Screening of sparkling wines for the presence of PAU5 revealed that those sparkling wines lacking PAU5 with only one exception showed gushing upon opening of the bottle. Compared to a situation in which no analytical means at all are available for gushing in sparkling wine, the finding will be regarded as a breakthrough from a manufacturer's point of view. Even if some of the gushing sparkling wines did contain PAU5, it must be considered that the occurrence of gushing can have a variety of causes like secondary gushing, as already discussed in section 4.1. In addition, the presence of fungal surface-active proteins like hydrophobins known as gushing inducers in beer were not determined in this thesis.

4.6. The foam stabilizing properties of PAU5

As described in section 0 sparkling wines lacking PAU5 are more susceptible to the occurrence of gushing.

PAU5 was isolated in its native, highly glycosylated form from a non-gushing sparkling wine in semi-preparative amounts and with a high purity. The addition of the purified PAU5 to grape juice resulted in a highly stable foam upon nitrogen perfusion. Consequently, it was concluded that PAU5-free grape juice showed that the protein has a strong foam-stabilizing effect in that medium. In contrast, the addition of the protein after treatment with proteinase K resulted in a foam stability that was comparable with the PAU5-free grape juice used as the negative control. Similar results were previously reported (Lao et al., 1999). Wine treated with hydrolytic enzymes showed a strong decrease in foam stability. The authors showed that the loss of foam stability was accompanied by degradation of macromolecules such as polysaccharides and, mainly, proteins.

To the best of our knowledge, our study is the first report on the isolation of a foam-stabilizing protein from sparkling wine and the analysis of its properties. Many studies have stated the importance of mannoproteins on foam quality (Blasco et al., 2011; Núñez et al., 2006; Vincenzi et al., 2014). As the protein PAU5 shares homologies with the cell wall Tir/Dan proteins and is also glycosylated (Luo & Van Vuuren, 2008; Viswanathan et al., 1994), the foam-stabilizing effect of this protein might be comparable to the mechanism stated for mannoproteins, due to the amphiphilic character enhanced by glycosylation (Blasco et al., 2011).

We demonstrated that the foam-stabilizing effect of PAU5 strongly depends on the amount added to the grape juice. Nevertheless, it has to be taken into account that grape juice differs in its composition from wine and sparkling wine in regard to quality and quantity of sugars, the presence of yeast derived proteins as well as the presence of ethanol, organic acids and carbon dioxide. Those factors are known to influence the foamability and foam stability of proteins (Andrés-Lacueva et al., 1996; Brierley et al., 1996; Cilindre et al., 2010). Consequently, the exact amount of PAU5 needed to achieve a foam-stabilizing effect in sparkling wines cannot be estimated from this experiment.

This foam stabilizing effect of PAU5 was confirmed by the fermentation experiments described in section 3.8.1. The fermentation of the healthy juice with one of the two yeast strains resulted in an increase of foam stability. This was attributed to the presence of foam

stabilizing proteins introduced by the yeasts such as the protein PAU5. A previous infection of the juice with *B. cinerea* results in a complete loss of the foam stabilizing effect and a complete vanishing of PAU5 that could not be prevented by sulfur treatment. The heat treatment of botrytized juice led to even higher amounts of PAU5 accompanied by a considerable increase of the foam stability (this observation will be discussed in section 4.9). Consequently, the foam stability is highly connected with the presence of PAU5.

The analysis of bentonite-treated white wines confirmed this observation. The wine with the highest concentration of PAU5 was found to have the most stable foam.

Since PAU5 has foam stabilizing properties it was assumed that has a preventive effect on the occurrence of gushing, similar to the mechanism described for ns-LTP1 in beer.

4.7. The foam stabilizing effect of the seripauperin familiy

An investigation of base wines regarding their amount of PAU5 and their foam stability showed that base wines lacking PAU5 show the most unstable foam upon nitrogen perfusion. Therefore, a foam stabilizing effect of PAU5 in base wine was suggested. The results confirm the observations made in grape juice. Here an addition of PAU5 to grapes juice resulted in an increase of foam stability that depended on the amount of PAU5 added.

However, it was not possible to draw a direct correlation between the PAU5 concentration and foam stability in base wines. This result indicated that beside the foam stabilizing protein PAU5 other proteins in the base wines may have been present that had an impact on foam formation and stabilization. The seripauperins comprise the largest gene family in *S. cerevisiae* with 24 members. The PAU genes share a high sequence homology from 82 - 100 % (Rachidi et al., 2000; Rossignol et al., 2003). The high similarity within the seripauperin family suggests that also other PAU proteins may have a positive impact on foam stabilization in wine and sparkling wine and should therefore be considered in future studies.

4.8. Reduced amounts of ns-LTP1 from *V. vinifera* in gushing sparkling wines

The ns-LTP1 from barley contributes to foam formation in beer after glycation during the brewing process (Jégou et al., 2000; Sorensen et al., 1993). Hippeli and Hecht (2008) detected reduced amounts of ns-LTP1 in gushing beer as compared to a non-gushing beer of comparable make.

Gushing and non-gushing sparkling wines were analyzed for their amount of the PR-protein ns-LTP1 during the current study. All samples were found to contain this protein, but in different concentrations. The samples were assigned either to the group of the gushing or non-gushing sparkling wines. The gushing samples were divided into sparkling wines lacking PAU5 and containing PAU5 suggesting that gushing sparkling wines containing PAU5 might be affected by secondary gushing. The results revealed that in general the non-gushing sparkling wines tend to have higher amounts of ns-LTP1. However, there was no significant difference detectable between the three groups. Therefore, this protein is not a useful marker for the occurrence or absence of gushing in sparkling wine like the protein PAU5. It should be investigated in further experiments if ns-LTP1 from *V. vinifera* plays a role in foam formation of sparkling wines as observed in beer.

Gushing research in beer showed that the level of gushing rather depends on the ratio of gushing inducing proteins and the gushing inhibiting protein ns-LTP1 than the absolute concentration of those proteins. That mechanism might also apply for gushing induction of sparkling wines (Lutterschmid et al., 2011).

4.9. Prevention of a botrytized induced protein degradation by heat treatment

The current study revealed that the infection of the grape juice with *B. cinerea* and a following incubation with yeast resulted in a loss of the protein PAU5 and a decreased concentration of the protein ns-LTP1. This observation is in accordance with the findings obtained by comparing wine made from botrytized and healthy grapes. Also here, reduced amounts of PAU5 and ns-LTP1 were found in the botrytized wine.

The reduced PAU5 and ns-LTP1 content found in our experiments is most probably due to the degradation of the protein by proteases previously secreted during growth of *B. cinerea*. This assumption is also backed by previous research. According to Ten Have et al. (2004), *B. cinerea* secretes a group of aspartic proteases that are still active at low pH levels. Our results showed that the addition of potassium metabisulfite to the infected grape juice did not prevent the degradation of PAU5 and ns-LTP1. Sulfites are common additives in wine making. Besides their antioxidant activity, they are also used as an antimicrobial agent (Ribéreau-Gayon et al., 2006). The presence of sulfites did not have a protective effect on protein degradation. It is assumed that the fungal proteases secreted in the juice are not affected by the sulfur addition. This assumption is confirmed by SDS-PAGE analysis.

Proteins present in the botrytized but not in the healthy juice were assumed to be of fungal origin and were still visible in the sulfur treated juice.

Heat treatment of the botrytized juice at 85 °C before the fermentation with the yeast led to even higher amounts of PAU5 after yeast fermentation than in the healthy controls treated in the same way. The amount of ns-LTP1 was only slightly reduced compared to the healthy control. We assume that *B. cinerea* related proteins responsible for protein degradation get inactivated at higher temperatures, accompanied by a loss of the viability of the fungus. This suggestion was confirmed by the results of the SDS-PAGE analysis of the respective juice. Protein bands present in the infected juice but not in the healthy juice without yeast treatment were assumed to be of fungal origin. However, the heat treatment of infected juice before yeast addition led to a complete loss of the proteins introduced by *B. cinerea*. We conclude that proteins expressed by *B. cinerea*, in particular aspartic proteases, are sensitive to higher temperatures. Ten Have et al. (2004) investigated the effect of the pH on the activity of proteases secreted by *B. cinerea*, but no explicit research was performed on the effect of temperature.

The increased concentration of PAU5 in the botrytized and heat-treated sample compared to the healthy control can be explained as follows: Protein degradation of grape proteins occurs in the botrytized juice before heat treatment induced by fungal enzymes. Heating up the botrytized material results in a depletion of fungal proteins as mentioned above, accompanied with the loss of their proteolytic activity. The cleavage products of the degraded proteins remain in the juice and may act as nitrogen and carbon sources, which are known as yeast nutritional compounds (Spencer, Spencer, & de Figueroa, 1997). A higher availability of those compounds in the juice may contribute to a higher viability of the yeast, resulting in a generally higher secretion of proteins, including PAU5.

4.10. Influence of the yeast strain on the PAU5 concentration

The two different *S. cerevisiae* strains used for fermentation secreted PAU5 in different amounts into the surrounding medium. Therefore, we suggest that the choice of yeast strain may have an impact on the foaming potential of wines and sparkling wines. This observation is in agreement with the findings of other studies that investigated the influence of the yeast strain used for fermentation regarding its foaming properties and the release of peptides and proteins (Martıinez-Rodrıiguez et al., 2001; Martinez-Rodriguez et al., 2002). A detailed

characterization of the role of the yeast strain used in the first and second fermentation in sparkling wine production regarding the PAU5 concentration remains to be elucidated.

4.11. Influence of bentonite treatment on the concentration of PAU5 and ns-LTP1

In this thesis it was demonstrated that the amount of the protein PAU5 from *S. cerevisiae* and ns-LTP1 from *V. vinifera* in wine can be affected by treatment with bentonite. This mineral absorbent is widely used in the treatment of food and beverages. In wine processing, it is frequently applied to remove non-thermostable proteins and polyphenols. The absorbent can be applied in different steps of the production process. Due to its known interaction with proteins, in this study its specific effect on the amount PAU5 and ns-LTP1 occurring in the final wine was investigated.

Both the type of bentonite and the stage of production during which it is added can influence the amount of PAU5 removal. The later the bentonite was applied during the production process, the more PAU5 was removed, even when the amount of bentonite used for the fining of the final wine was reduced to 250 g/hl instead of the usual 300 g/hl used in earlier fining treatments. Even if the bentonite was present during the whole fermentation process, when added after pre-clarification there was no significant difference in the final PAU5 concentration compared to the untreated control. The fining of the must itself resulted in one case in even higher amounts of PAU5 in the finished wine and in the wine with the highest foam stability. The bentonite added to the must was separated from the must in the pre-clarification process before yeast addition. Consequently, the yeast protein PAU5 cannot adsorb to the bentonite. Nevertheless, SDS-PAGE revealed that the bentonite treatment of the must affects the electrophoretic pattern of the wines and therefore its protein composition. The removal of proteolytic proteins from the must might be a possible reason for elevated PAU5 concentrations in the wine.

The bentonite-induced removal of ns-LTP1 followed another scheme. The time of addition in the production process turned out to have a higher impact on the ns-LTP1 concentration than the type of bentonite applied. The most ns-LTP1 was removed when the bentonite was added after the pre-clarification resulting in the wine with the most unstable foam.

The removal of proteins from wine and sparkling wine by the adsorption to bentonite and its influence on foam quality was already discussed in the literature. Vanrell et al. (2007) found a diminished foamability in sparkling wine accompanied by the removal of wine proteins

using bentonite as a riddling agent. A reduced foam quality and content of total protein of Champagne base wines were observed by Marchal et al. (2002) after treatment with bentonite.

However, the current study revealed that the processing stage during which bentonite treatment was applied influences the removal of proteins, both quantitatively and qualitatively. Beside the process stage, the study showed that the brand of bentonite used seems to have an impact on the amount of PAU5 being removed from the process. Commercial bentonite consists of a negatively charged clay mineral with montmorillonite as the main component. The particular affinity of proteins to bentonite is influenced by physiochemical molecular interactions such as cation-exchange, hydrophobic or hydrophilic interactions, van der Waal interactions or hydrogen-bonding mechanisms (Blade & Boulton, 1988; Gougeon et al., 2002; Gougeon, Soulard, Reinholdt, et al., 2003; Staunton & Quiquampoix, 1994). Consequently, the protein-bentonite interactions depend on factors such as the net charge, molecular weight, and exposure of hydrophilic or hydrophobic parts of the protein. The net charge of a protein is influenced by its isoelectric point and the pH of the surrounding medium. The exposure of specific protein parts depends on its tertiary conformation. External factors such as ionic strength or ethanol concentration have an impact on protein conformation.

In addition the properties of the bentonite itself influence the protein-bentonite interaction. Gougeon, Soulard, Miehé-Brendlé, et al. (2003) showed a more effective protein removal by the enrichment of bentonite with Na^+ ions. These results are in agreement with those findings since different proteins were affected differently by bentonite treatment due to their physicochemical properties. Additionally, the processing stage in which bentonite was added affected the protein removal. During the wine making process, the surrounding medium is constantly changing in factors such as pH, ionic strength or ethanol concentration. All these factors influence the proteins and their adsorption to bentonite. However, the second fermentation in sparkling wine production was not considered in this study and remains to be elucidated.

5. SUMMARY

Spontaneous over-foaming of carbonated beverages after pressure release upon opening the bottle is referred to as gushing. This unwanted phenomenon results in adverse financial consequences and an image loss for the affected manufacturers. The reasons for the development of this phenomenon in sparkling wine have hardly been investigated.

Therefore, the aim of the study was to elucidate gushing influencing factors in sparkling wine with emphasis on yeast and grape derived proteins.

The current work revealed that in some gushing sparkling wine and carbonated grape juice samples there were similarities detected with the secondary type of gushing described in beer. Here the nucleation sites for bubble growth that contributed to the unwanted over-foaming were found to be crystals, residual filter aids and protein haze.

For a closer examination of gushing influencing proteins, a new method for protein purification of grape derived beverages and grapes was developed. SDS-PAGE and RP-HPLC were applied to analyze the protein composition of healthy and botrytized grapes and of wines made from botrytized and healthy grapes. *B. cinerea* infection led to a general decrease of protein content in infected grapes and wines suggesting proteolytic activity of this fungus. Especially the concentration of a protein which was identified as Seripauperin 5 (PAU5) from *S. cerevisiae* underwent a significant decrease in wine made from infected grapes.

A degradation of PAU5 and other proteins and the occurrence of a laccase from *B. cinerea* were observed in a gushing sparkling wine. Screening of sparkling wines showed that samples lacking PAU5 had a high probability for the occurrence of gushing.

The protein PAU5 was found to have foam-stabilizing properties and may thus have a direct influence on the gushing potential of sparkling wines. Since the seripauperin family shares a high sequence homology, it was concluded that other PAU proteins have an impact on foam stability in wine.

To elucidate whether the protein ns-LTP1 from the grape has also an impact on the development of gushing similar to the mechanism described for beer, western blot and ELISA assays were developed and applied. Reduced amounts of ns-LTP1 were detected in gushing sparkling wines but there was no direct correlation between the occurrence of gushing and the concentration of ns-LTP1. An infection of the grapes with *B. cinerea* resulted

in reduced amounts of ns-LTP1 in the grapes as well as in the resulting wine suggesting that this protein is susceptible to fungal proteases.

The fermentation experiments carried out with grape juice revealed that unlike sulfur treatment, heat treatment prevented the protein degradation of ns-LTP1 and PAU5 induced by *B. cinerea* and resulted in even higher amounts of PAU5 compared to the juice fermented with yeast without a previous botrytization. In addition, it was shown that the yeast strain used for fermentation has an impact on the amount of PAU5 released into the surrounding medium.

In further experiments, the fining process of the wine with bentonite was examined for its potential to remove PAU5 and ns-LTP1 from the wine. RP-HPLC analysis and ELISA of wines revealed that bentonite treatment affected as well PAU5 as ns-LTP1 concentrations in the final product. The extent of PAU5 removal depended on both the type of bentonite applied as well as on time of addition in the production process. The amount of ns-LTP1 removed from the wine was more influenced by the time of addition than the type of bentonite applied.

6. ZUSAMENFASSUNG

Ein heftiges Überschäumen von karbonisierten Getränken direkt nach dem Öffnen wird als Gushing bezeichnet. Dieses Phänomen hat keinen Einfluss auf die sensorischen Eigenschaften eines Getränkes, wird aber von den Kunden als ein Produktfehler wahrgenommen. Dies führt zu einem Imageverlust der betroffenen Produzenten, welches wiederum finanzielle Nachteile zur Folge hat. Aufgrund dessen sind die Hersteller bemüht die Entstehung von Gushing zu verhindern. Allerdings sind die Ursachen für das unerwünschte Überschäumen in Schaumweinen noch weitestgehend unerforscht.

Das Ziel dieser Arbeit war es gushing-beeinflussende Substanzen, mit einem Fokus auf Planzen- und Hefeproteine, in Schaumwein zu identifizieren.

In dieser Arbeit wurde in gushenden Schaumwein- und Saftproben Parallelen zu dem sekundären Gushing, das in Bier beschrieben ist, gefunden. Hier wurden Kristalle, Filtermittelrückstände und Eiweißtrübungen als mögliche Nukleationskeime, die in einer exzessiven Blasenbildung resultierten, identifiziert.

Um die Rolle von oberflächenaktiven Proteinen bei der Entstehung von Gushing in Schaumwein genauer zu untersuchen, wurde in dieser Arbeit eine entsprechende Methode zur Aufreinigung von Proteinen aus Trauben, Wein und Schaumwein entwickelt. Daraufhin wurde mittels SDS-PAGE Analyse und RP-HPLC gesunde und mit *B. cinerea* infizierte Trauben sowie die korrespondierenden Weine untersucht. Eine Infektion der Trauben mit dem Schimmelpilz *B. cinerea* führte sowohl in der Traube selbst als auch in dem entsprechenden Wein zu einer Abnahme der Proteindiversität und –quantität, welches auf die proteolytische Aktivität dieses Pilzes zurückgeführt wurde. Das Protein Seripauperin 5 (PAU5) aus *S. cerevisae*, lag im botrytisierten Wein in einer signifikant geringeren Konzentration vor.

Ein ähnliches Resultat zeigte die Untersuchung von einem gushenden und nicht-gushenden Schaumwein. Die gushende Probe wies eine geringere Proteindiversität und –quantität auf. Desweitern konnte in der gushenden Probe eine Laccase von *B. cinerea* identifiziert werden. Ein Screening weiteren Schaumweinen zeigte, dass die Proben, in welchen kein PAU5 detektiert wurde zu einer sehr hohen Wahrscheinlichkeit von Gushing betroffen sind.

Es konnte gezeigt werden, dass das Protein PAU5 schaumstabilisierende Eigenschaften besitzt und somit möglicherweise einen direkten Einfluss auf das Gushingpotential von Schaumweinen hat. Da es innerhalb der Proteinfamilie der Seripauperine sehr viele Gemeinsamkeiten gibt wurde angenommen, dass auch weiter PAU Proteine einen Einfluss auf die Schaumstabilität haben.

Mittels Western blot Analyse und ELISA wurden Trauben, Weine und Schaumweine auf die Menge an dem Pflanzenprotein ns-LTP1 von *V. vinifera* untersucht, um etwaige Parallelen mit der Gushing Forschung in Bier aufzudecken. In gushenden Schaumweinen wurden geringere ns-LTP1 Konzentrationen gefunden. Allerdings konnte keine direkte Korrelation zwischen dem Auftreten von Gushing und der Konzentration an ns-TLP1 in Schaumweinen festgestellt werden. Eine Infektion der Trauben mit *B. cinerea* führte sowohl in der Traube selbst als auch im korrespondierende Wein zu einer Konzentrationsabnahme von ns-LTP1. Aufgrund dessen wurde angenommen, dass dieses Pflanzenprotein nicht resistent gegenüber pilzlichen Proteasen ist.

Die mit Traubensaft durchgeführten Fermentationsexperimente zeigten, dass eine Hitzebehandlung den Botrytis-induzierten ns-LTP1- und PAU5-Abbau stoppen kann. Die Hitzebehandlung von botrytisierten Material führte zu sogar höheren Konzentrationen an PAU5. Die Zugabe von Schwefel hatte keinen inhibierenden Einfluss auf den Proteinabbau. Ein Vergleich von zwei verschiedenen *S. cerevisiae* Stämmen zeigte, dass diese unter identischen Bedingungen, unterschiedliche Mengen an PAU5 exprimierten.

Die Analyse von Bentonit-behandelten Weinen zeigte, dass die Konzentration von ns-LTP1 und PAU5 im fertigen Produkt durch die Anwendung von Bentonit beeinflusst werden kann. Es konnte gezeigt werden, dass sowohl der Zugabezeitpunkt im Produktionsprozess als auch der verwendete Bentonittyp sich unterschiedlich stark auf die Menge an PAU5, die entfernt wurde, auswirkten. Bei der Entfernung von ns-LTP1 hingegen zeigte sich, dass der Zugabezeitpunkt im Produktionsprozess eine größere Rolle spielt als der verwendete Bentonittyp.

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8. APPENDIX

Table 14: Proteins identified in the sparkling wines via nano-ESI-LC-MS/MS

Exp MW[1]	Th MW[2]	identified protein (organism)	accession	Cov[3] [%]	MS[4]	emPAI	total peptide count and sequence	unique peptide count and sequence
13	12.93	unnamed protein (*V. vinifera*)	CBI35210	49.7	657	1.20	7	7
								(R)SKYGWTAFCGPSGPTGQAACGK(C)
								(K)YGWTAFCGPSGPTGQAACGK(C)
								(K)CLSVTNTATGTQATVR(I)
								(S)VTNTATGTQATVR(I)
								(R)SKYGWTAFCGPSGPTGQAA(C)
								(R)IVDQCSNGGLDLDSGVFNQ(L)
								(K)GHLTVNYQFVNCGD(-)
Exp MW[1]	Th MW[2]	identified protein (organism)	accession	Cov[3] [%]	MS[4]	emPAI	total peptide count and sequence	unique peptide count and sequence
18	12.93	unnamed protein (*V. vinifera*)	CBI35210	36.4	207	1.20	4	4
								(R)SKYGWTAFCGPSGPTGQAACGK(C)
								(K)YGWTAFCGPSGPTGQAACGK(C)
								(K)CLSVTNTATGTQATVR(I)
								(K)GHLTVNYQFVNCGD(-)
Exp MW[1]	Th MW[2]	identified protein (organism)	accession	Cov[3] [%]	MS[4]	emPAI	total peptide count and sequence	unique peptide count and sequence
25	21.25	hyp. protein (*V. vinifera*)	CAN66515	49.3	693	1.74	28	6
								(K)TDEYCCNSGSCNATTYSEFFK(T)
								(N)SGSCNATTYSEFFK(T)
								(G)SCNATTYSEFFK(T)
								(K)TRCPDAYSYPKDDQTSTFTCPSGTN(Y)
								(K)TRCPDAYSYPKDDQTSTFTCPSGTNYEVIF(C)
								(R)CPDAYSYPKDDQTSTFTCPSG(T)
							(K)TRCPDAYSYPK(D)	
							(K)TRCPDAYSYPKDDQTSTFT(C)	
							(R)CPDAYSYPK(D) (R)CPDAYSYPKDDQ(T)	
							(R)CPDAYSYPKDDQTSTF(T)	
							(R)CPDAYSYPKDDQTSTFT(C)	
							(Q)SWSLNVNAGTTGGR(V)	
							(W)SLNVNAGTTGGR(V)	
							(R)TNCNFDASGNGK(C)	
							(R)TNCNFDASGNGKCETG(D)	
							(R)GISCTADIVGECPAALK(T)	
							(R)GISCTADIVGECPAALKTTGG(C)	
							(R)GISCTADIVGECPAALKTTGGCNNPCTVFK(T)	
							(G)ISCTADIVGECPAALK(T)	
							(C)TADIVGECPAALK(T)	
							(K)TTGGCNNPCTVFK(T)	
							(K)TTGGCNNPCTVFKTDEYC(C)	

							(K)TTGGCNNPCTVFKTDEY CCNSG(S)	
							(T)TGGCNNPCTVFK(T)	
							(T)GGCNNPCTVFK(T)	
							(G)GCNNPCTVFK(T)	
Exp MW[1]	Th MW[2]	identified protein (organism)	accession	Cov[3] [%]	MS[4]	emPAI	total peptide count and sequence	unique peptide count and sequence
26	21.25	hyp..protein (*V. vinifera*)	CAN66515	59.10	703	1.42	35	5
								(D)GFNVAMAFNPTSNGCT R(G)
								(N)VAMAFNPTSNGCTR(G)
								(K)TDEYCCNSGSCNATTYS EFFK(T)
								(K)TRCPDAYSYPKDDQTST FTCPSG(T)
								(R)CPDAYSYPKDDQTSTFT CPSG(T)
							(K)TRCPDAYSYPK(D)	
							(K)TRCPDAYSYPKD(D)	
							(R)CPDAYSYPK(D)	
							(R)CPDAYSYPKD(D)	
							(R)CPDAYSYPKDDQT(S)	
							(R)CPDAYSYPKDDQTSTF(T)	
							(R)CPDAYSYPKDDQTSTFT(C)	
							(G)GGMQLGSGQSWSLNVN AGTTGGR(V)	
							(Q)SWSLNVNAGTTGGR(V)	
							(W)SLNVNAGTTGGR(V)	
							(S)LNVNAGTTGGR(V)	
							(R)TNCNFDASGNGK(C)	
							(R)TNCNFDASGNGKCETG(D)	
							(R)TNCNFDASGNGKCETGD(C)	
							(R)TNCNFDASGNGKCETGD CG(G)	
							(A)MAFNPTSNGCTR(G)	
							(R)GISCTADIVGECPAALK(T)	
							(R)GISCTADIVGECPAALKT TGGCNNPCTVFK(T)	
							(S)CTADIVGECPAALK(T)	
							(C)TADIVGECPAALK(T)	
							(T)ADIVGECPAALK(T)	
							(A)DIVGECPAALK(T)	
							(K)TTGGCNNPCTVFK(T)	
							(K)TTGGCNNPCTVFKTD(E)	
							(K)TTGGCNNPCTVFKTDEY C(C)	
							(T)TGGCNNPCTVFK(T)	
							(T)GGCNNPCTVFK(T)	
							(G)GCNNPCTVFK(T)	
							(R)CPDAYSYPKDD(Q)	
							(R)CPDAYSYPKDDQTS(T)	
Exp MW[1]	Th MW[2]	identified protein (organism)	accession	Cov[3] [%]	MS[4]	emPAI	total peptide count and sequence	unique peptide count and sequence
> 42	61.43	lcc2, laccase (*B. cinerea*)	CCD44233	19.4	478	0.74	13	13
								(G)PATADYDEDVGAIFLQ DWAHK(S)
								(K)SVFEIWDSAR(Q)
								(K)KFELTFVEGTK(Y)
								(K)KFELTFVEGTKYR(L)
								(K)FELTFVEGTK(Y)
								(K)FELTFVEGTKYR(L)
								(R)YDVIVEANAAADNYWI R(G)
								(R)GNWGTTcSSNSEAANA

								TGILR(Y)
								(R)YDSSSTVDPTSVGVTPR(G)
								(D)SSSTVDPTSVGVTPR(G)
								(K)SPANFNLVNPPR(R)
								(K)SPANFNLVNPPRR(D)
								(N)GPATADYDEDVGAIFLQDWAHK(S)
Exp MW[1]	Th MW[2]	identified protein (organism)	accession	Cov[3] [%]	MS[4]	emPAI	total peptide count and sequence	unique peptide count and sequence
18	11.09	PAU5 (S. cerevisiae)	KZV11581	59.0	478	1.03	14	9
								(A)AGASAAATTTLSQSDER(V)
								(A)GASAAATTTLSQSDER(V)
								(A)SAAATTTLSQSDER(V)
								(S)AAATTTLSQSDER(V)
								(A)AATTTLSQSDER(V)
								(A)ATTTLSQSDER(V)
								(A)TTTLSQSDER(V)
								(R)VITGVPWYSSR(L)
								(R)LKPAISSALSADGIYTIAN(-)
							(R)VNLVELGVYVSDIR(A)	
							(L)GVYVSDIR(A)	
							(M)LTGIPADQVTR(V)	
							(L)TGIPADQVTR(V)	
							(T)GIPADQVTR(V)	
Exp MW[1]	Th MW[2]	identified protein (organism)	accession	Cov[3] [%]	MS[4]	emPAI	total peptide count and sequence	unique peptide count and sequence
18	11.09	PAU5 (S. cerevisiae)	KZV11581	59.0	394	1.57	13	8
								(A)AGASAAATTTLSQSDER(V)
								(A)GASAAATTTLSQSDER(V)
								(A)SAAATTTLSQSDER(V)
								(A)AATTTLSQSDER(V)
								(A)ATTTLSQSDER(V)
								(A)TTTLSQSDER(V)
								(R)AHLAEYYSF(Q)
								(R)LKPAISSALSADGIYTIAN(-)
							(R)VNLVELGVYVSDIR(A)	
							(L)GVYVSDIR(A)	
							(M)LTGIPADQVTR(V)	
							(T)GIPADQVTR(V)	
							(G)IPADQVTR(V)	

[1]Exp MW = apparent molecular weight due to SDS-PAGE analysis; [2]Th MW = Theoretical molecular weight, calculated based on the amino acid sequence without signal sequence; [3]Cov = percentage sequence coverage; [4]MS = Mascot Score

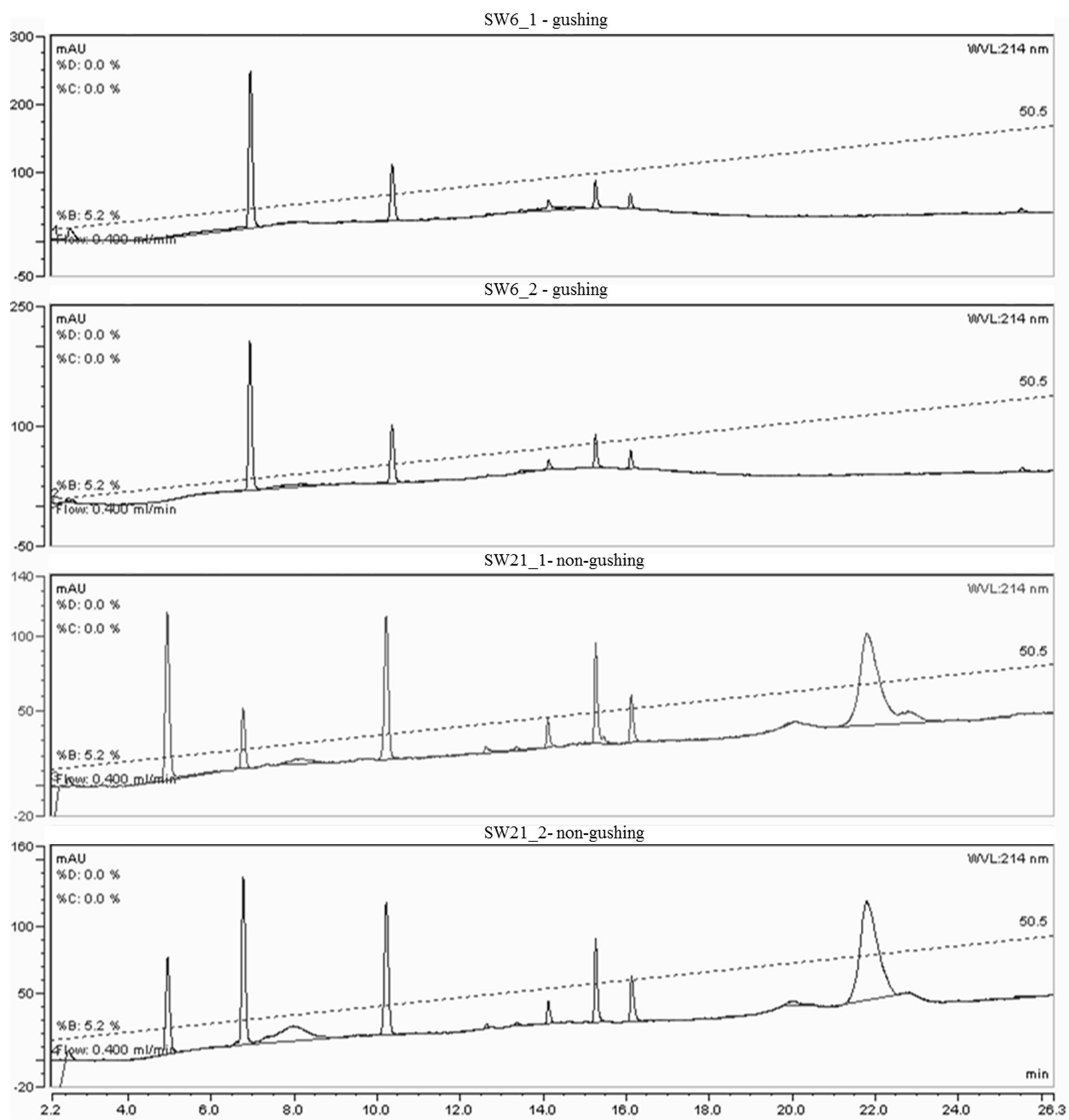

Figure 37: RP-HPLC chromatograms of a gushing sparkling wine (SW6) and a non-gushing sparkling wine (SW21) in technical replicates

9. List of publications and student theses

Peer-reviewed journals

Kupfer, V.M., Vogt, E.I. Siebert, A.K., Meyer M.L., Vogel, R.F. Niessen, L.: Foam-stabilizing properties of the yeast protein PAU5 and evaluation of factors that can influence its concentration in must and wine, Food Research International, 2017; 102; 111-118

Kupfer, V.M., Vogt, E.I., Ziegler, T., Vogel, R.F., Niessen, L.: Comparative protein profile analysis of wines made from Botrytis cinerea infected and healthy grapes reveals a novel biomarker for gushing in sparkling wine, Food Research International, 2017; 99 (1); 501-509

Vogt, E.I., Kupfer, V.M., Vogel, R.F., Niessen, L.: Überschäumenden Sekt auf der Spur, Der Deutsche Weinbau, 2017; 11; (29-35)

Vogt, E.I., Kupfer, V.M., Vogel, R.F., Niessen, L.: Evidence of gushing induction by Penicillium oxalicum proteins, Journal of Applied Microbiology, 2016; 122 (3); 708-718

Vogt*, E.I., Kupfer*, V.M., Vogel, R.F., Niessen, L.: A novel preparation technique of red (sparkling) wine for protein analysis, EuPA Open Proteomics, 2016; 11; 16-19
*joint first authorship

Oral presentations

Kupfer, V.M., Einsatz bioanalytischer Methoden zur Identifizierung von gushing-relevanten Markerproteinen in Wein und Schaumwein, Jahrestagung des Forschungsrings des Deutschen Weinbaus, Forschungsring des Deutschen Weinbaus, 29.04.2016, Bad Kreuznach, Germany

Kupfer, V.M., Vogt, E.I., Einfluss von oberflächenaktiven Proteinen auf das Gushing von Sekt und Schaumwein, Mitgliederversammlung des Verbandes Deutscher Sektkellereien e.V., 29.04.2015, Wachenheim, Germany

Poster Presentations

Kupfer, V.M., Vogt, E.I., Ziegler, T., Vogel, R.F., Niessen, L.: Comparative proteomic analysis of wines made from *Botrytis cinerea* infected and healthy grapes reveals interesting parallels to the gushing phenomenon in sparkling wine, presented at "Macrowine 2016", 25.062016 – 30.06.2016, Nyon, Switzerland

Vogt, E.I., Kupfer, V.M., Vogel, R.F., Niessen, L.: Influence of *Penicillium oxalicum* proteins on gushing of sparkling, presented at "Macrowine 2016", 25.062016 – 30.06.2016, Nyon, Switzerland

Kupfer, V.M., Vogt, E.I., Vogel, R.F., Niessen, L.: Preparation of red sparkling wine for protein analysis by SDS-PAGE and MALDI-TOF MS, presented at "In Vino Analytica Scentica 2015", 14.07.2015 – 17.07.2015, Mezzocorona, Italy

Student Theses

The following student theses were supervised by the author. The obtained raw data were partially incorporated into this thesis with permission by the respective students.

Master Thesis:

Siebert Annemarie K., Identifizierung und Charakterisierung von Gushing assoziierten Proteinen im Schaumwein, 2017

Research internship:

Ziegler Tobias, Einfluss von *Botrytis cinerea* auf das Proteom von Trauben und Wein, 2015

10. Curriculum Vitae

Work experience

Technical University Munich	**Scientific employee**
Department of Technical Microbiology	Applied research; guidance of students, project-related professional exchange with industrial partners and the scientific community, microbiology, protein purification, analytics (SDS-PAGE, HPLC, MALDI-TOF MS, Western blot, ELISA)
05.2014 - 06.2017	
Max-Planck Institute for Psychiatry	**Internship – Diploma thesis**
Ag Chemical Genomics	Basic medical research; cell culture, protein purification, analytics (SDS-PAGE, Western blot, radioactive-ligand binding assay)
10.2012 - 03.2013	
Microcoat Biotechnologie GmbH	**Internship**
09.2011 - 01.2012	R&D in GLP environment; creating and application of SOPs, protein purification, development of nanogold based lateral flow immunoassays, analytics (SDS-PAGE, HPLC)
Max-Planck Institute for Psychiatry	**Internship**
Ag Chemical Genomics	Basic medical research; protein engineering (cloning, PCR, primer design, site directed mutagenesis), protein purification, analytics (SDS-PAGE, Western blot, fluorescence-ligand binding assay)
03.2010 - 07.2010	

Education

Technical University Munich	**Doctorate (Dr. rer. nat.)**
Department of Technical Microbiology	The influence of plant- and yeast-derived proteins on gushing in sparkling wine
05.2014 – 22.01.2018	
University of applied Sciences Weihenstephan-Triesdorf	**Studies of Biotechnology; graduated as Diplom Ingenieur (FH)**
10.2007- 07.2013	Final grade: 1,4 Diploma thesis: Biochemical characterization of the interaction between the glucocorticoid receptor and the FK506 binding proteins FKBP51 and FKBP52 Focus of studies: process engineering, biotechnological production processes, biochemistry, analytics
Dom-Gymnasium Freising	**Higher Education Entrance Qualification**
09.1998 - 06.2007	