It’s time to pop a cork on champagne’s proteome!☆

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1. Introduction

Champagne, the world-renowned French sparkling wine, is a multicomponent hydroalcoholic system holding a huge quantity of various compounds. Proteins are not the major components of wine, although they are essential compounds, contributing to many organoleptic characteristics. Indeed, wine proteins are implied in the foaming properties of Champagne and sparkling wines [1–3], the interaction with wine volatile compounds [4], the stabilization of tartaric salts [5], the decrease in wine astringency [6] and, unfortunately, the formation of haze in white wines [7].

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Since the end of the 90s, a broad range of methods for the separation of specific or total proteins have been developed and applied to wine [8], including chromatographic techniques [9–12], capillary gel electrophoresis [13], one-dimensional polyacrylamide gel electrophoresis [1,7,11], or two-dimensional electrophoresis [3,7,15–17]. Despite these complementary approaches applied to depict the wine proteome, only few different protein species – from 4 to 15 – were identified in still wines, but none were reported in Champagne wines. In 2006, Okuda et al. [17] have undergone successive steps to extract the soluble proteins from a Chardonnay wine. Though more than 300 spots were visualized on Coomassie Brilliant Blue (CBB-R250) stained 2D-gels, only 4 different grape protein species were identified by N-terminal sequencing. In 2008, 14 different protein species originating mainly from *Vitis vinifera*, from *Saccharomyces cerevisiae* and *Botrytis cinerea* were undoubtedly identified in a Champagne Chardonnay base wine, by 2D-nano-LC–MS/MS and Western blot analysis [16]. Most of these identified grape proteins were also isolated in a Semillon wine by hydrophobic interaction chromatography [11]. More recently, Sauvage et al. (2010) [7] employed 2D electrophoresis to monitor the impact of enological treatments (bentonite) on the protein content of a Chardonnay wine. Only ten spots were displayed, corresponding to seven protein species from grape origin exclusively. These previous studies illustrate the complexity of wine protein extraction and analysis, owing to the numerous methods available. On the other hand, various factors might explain the qualitative and quantitative changes of the wine protein content, as for example: the grape variety [14,15], the infection of grape berries by the widespread phytopathogen *B. cinerea* [3,16], the yeast strain employed for alcoholic fermentation [18], the aging on lees [19], and also various fining treatments applied during the winemaking process [1,7,9]. It might be thus important to prefer a short extraction protocol suitable for a low protein content (as this is the case for wine) that allows the identification of as many proteins as possible without causing degradation and/or chemical modification.

Combinatorial Peptide Ligand Libraries (CPLLs) have been first proposed, in 2010, to depict the proteome of red and white Italian still wines [20,21]. This technology has been successfully applied to various beverages and foods (from animal and plant sources) and allows the detection of low abundance protein species in comparison with classical methods (as mentioned earlier) [22,23]. Among the main advantages of this new technology, CPPLs allowed the detection of exogenous wine proteins in commercial wines, such as: milk allergen proteins [20] or fungal proteins linked to a potentially contaminated harvest [21]. In white wines, Cereda et al. [20] have detected trace amounts of casein, a fining agent, with a limit of detection equal to 1 μg/L for casein, thus 250 times more sensitive than official ELISA methods (as recommended by OIV for routine controls). Anyway, this method permitted the identification of more than 100 unique gene products in a white wine (not treated with fining agents, though) [22] and around 25 proteins in a Valpolicella red wine [21], much more than in previous studies.

While proteins are generally considered as being important sparkling wine components for the stabilization of their foaming properties, no studies have clearly identified, to date and to our knowledge, the proteins from champagne. Indeed, most studies were dedicated to Champagne base wine proteins [1,3,16]. Champagne wines (and some other French and foreign sparkling wines) are elaborated through the traditional method, which consists in two major yeast-fermented steps, to transform sugars into ethanol and gaseous CO_2: (i) a first alcoholic fermentation (from grape must to base wine), and (ii) a second alcoholic fermentation in sealed bottle, the so-called "prise de mousse" (from base wine to champagne). The second alcoholic fermentation (6–8 weeks) and the maturation on yeast lees (which may last from 12 months up to several years) both induce various quantitative and qualitative changes in the wine through the action of yeast, as listed hereafter: (1) development of aromas during aging on lees, (2) release of nitrogen compounds during autolysis, and (3) release of macromolecules (polysaccharides, lipids, nucleic acids) in wine [24]. Champagne wines are elaborated through a long winemaking process, leading to an unavoidable loss of proteins. In 2003, Manteau et al. [25] demonstrated, by SDS-PAGE and Western blot, the complete extinction of proteins through the winemaking process of a single-varietal champagne elaborated with the Pinot Noir variety. Nevertheless, these results were not confirmed by Le Bourse et al. [26] who revealed by SDS-PAGE the presence of two bands at 60 and 18 kDa in a champagne made with Chardonnay. Unfortunately, these authors did not identify these champagne proteins.

In the present study, we applied the powerful CPLL technique to provide the first insight of the low-abundance proteome from two representative types of Champagne wines made from either 100% Chardonnay (single-varietal champagne, "*Blanc de blancs*") or a blend of three grape varieties (Chardonnay, Pinot noir and Pinot Meunier). The main advantage of CPLLs, as compared to previous extraction procedures, is the detection of low abundance proteins despite the absence of purification or pre-concentration step. It is an important fact to take into account, since Champagne wines generally contain a low amount of proteins (5–10 mg/L) that implies to usually concentrate wine proteins before 1D or 2D electrophoresis.

2. Material and methods

2.1. Champagne wines

Two commercial Champagne wines elaborated, in 2011, at the Nogent l'Abbesse Cooperative (Marne, France) were used for this set of experiments. These Champagne wines underwent the same traditional winemaking but differed from their grape variety content. A first type of champagne, a single-varietal champagne (also named "*Blanc de Blancs*") was only elaborated with base wines from white Chardonnay grapes, whereas the second batch, a blended champagne ("*Brut non-vintage*") was a mixture of base wines from all three grape varieties: Chardonnay (32%), Pinot Noir (19%) and Pinot Meunier (49%). These two types of Champagne wines are the most representative, though *Blanc de Blancs* has a higher consumer preference. Both champagnes underwent malolactic fermentation. No fining treatment was applied during the vinification process, except two riddling agents containing sodium bentonite and tannins added to the

tirage solution (with yeast and sucrose) in order to help the sedimentation of yeast lees before disgorging.

2.2. Protein extraction

For each Champagne wine, the entire content of a bottle (750 mL) was first degassed under reduced pressure and stirring for a couple of hours. The protein capture was performed by mimicking reversed-phase conditions at pH 2.2 in the presence of 0.1% TFA as ion-pairing agent, via Combinatorial Peptide Ligand Libraries (CPLLs). The capture was implemented by adding 100 μL of CPLLs to 750 mL of champagne, via gentle shaking for 3 h at room temperature; then the beads were collected by filtration. The captured proteins from the peptide library were then desorbed by using a solution composed of 4% SDS and 2% DTT for 15 min, under boiling conditions. Desorption was carried out twice with 100 μL each and the two eluates pooled. Such extractions were done in duplicate with six bottles of Champagne wine.

2.3. SDS-PAGE and protein identification

The samples were loaded onto the SDS-PAGE gel, composed by a 4% polyacrylamide stacking gel (125 mM Tris–HCl, pH 6.8, 0.1% SDS) cast over a 8–18% resolving polyacrylamide gel (in 375 mM Tris–HCl, pH 8.8, 0.1% SDS buffer). The cathodic and anodic compartments were filled with Tris–glycine buffer, pH 8.3, containing 0.1% SDS. Electrophoresis was run at 100 V until the dye front reached the bottom of the gel. Staining and distaining were performed with colloidal Coomassie Blue and 7% acetic acid in water. The lanes of SDS-PAGE of all eluates were trypsin digested as previously described [21].

The tryptic mixtures were acidified with formic acid up to a final concentration of 10%. Eight microliters of tryptic digest for each band was injected in a nano-chromatographic system, UltiMate 3000 RSLCnano System (Thermo Scientific). The peptide mixtures were loaded on a reversed-phase trap column (Acclaim PepMap100, C18, 100 Å, 100 μm i.d. × 2 cm, Thermo Scientific) for the cleanup and pre-concentration. After cleanup, the valve was switched to place the trap column in series with a fused silica reverse-phase column (picoFrit column, C18, 2.7 μm, New Objective). The peptides were eluted with a 30 min gradient from 4% buffer A (2% acetonitrile and 0.1% formic acid in water) to 60% buffer B (2% water and 0.1% formic acid in acetonitrile) at a constant flow rate of 300 nL/min. The liquid chromatography was connected to an LTQ-XL mass spectrometer (Thermo Scientific) equipped with a nano-spray ion source. Full scan mass spectra were acquired in the mass range m/z 350 to m/z 2000 Da and the five most intense ions were automatically selected and fragmented in the ion trap. Target ions already selected for mass spectrometry (MS/MS) were dynamically excluded for 30 s. The raw data were analyzed separately by Mascot search engine (version 2.3.01), using Proteome Discover software (v.1.2.0 Thermo) and consulting first of all the contaminant database (version 13/05/2010, downloaded from http://maxquant.org/downloaded.html) to eliminate protein interference problems. Then the unassigned signals were searched by consulting the Uniprot_Viridiplantae database (30264 sequences, 184678199 residues) and Uniprot_*Saccharomyces cerevisiae* database (9832 sequences, 4551457 residues). Oxidation of methionine residues was set as a variable modification; two missed cleavages were allowed to trypsin; and peptide mass tolerance was set to 1 Da, fragment mass tolerance to 0.8 Da, and an ion source cut-off of 20 was chosen. The false discovery rate obtained by Proteome Discoverer, consulting the Mascot decoy database, was less than 0.01.

N-glycosylation sites were predicted by using the N-GlycoSite tool [27]. All MS data, reported in Tables 1, 2, 3 and 4, were only those found in at least five of six analyzed bottles.

3. Results and discussion

Fig. 1 displays the SDS-PAGE profiles of proteins from a single-varietal and a blended champagne (i.e., *Blanc de blancs* and Brut, respectively). Champagne proteins were extracted under the same conditions by CPLLs, and various volumes of eluates were loaded on SDS-PAGE gels. Lanes A and B correspond to 80 μL of eluate, whereas lanes E and F correspond to 25 μL of eluate. 80 μL of untreated champagne samples was also loaded on the same gel (lanes C and D) and did not show any protein band. Champagne wines usually have a low amount of proteins, around 5–10 mg/L, thus an optimal extraction procedure should be employed to visualize more proteins by SDS-PAGE and colloidal Coomassie Blue staining. Anyway, fewer protein bands, together with a lower intensity, were observed in the single-varietal champagne (lanes A and E), as compared to the blended champagne (lanes B and F). Eight and 9 gel segments were excised from the single-varietal champagne and the blended champagne, respectively, and submitted to MS analysis.

A total of 43 unique gene products were identified from both Champagne wines, among which 15 are common to both samples. All proteins were retrieved from Viridiplantae organisms (Tables 1, 2 and Supplementary Tables 1 and 2) and Fungi (Tables 3, 4 and Supplementary Tables 3 and 4); among them 12 belonged to *V. vinifera* and 7 to *S. cerevisiae* (the remaining part belonging to other Viridiplantae organisms). We report in Tables 1, 2, 3 and 4, only 23 proteins while the remaining 20 proteins (those identified with only 1 peptide, which might represent ultra-trace proteins visible only via the CPLL capture), are listed in the Supplementary Material. Even if many grape proteins were unambiguously identified according to the high number of peptides, most of them were considered as "putative uncharacterized" grape proteins (9 proteins) (Tables 1 and 2). On the other hand, a high number (24) of proteins from other Viridiplantae organisms were identified in both Champagne wines.

It has been previously demonstrated that Champagne base wines (i.e., wines obtained after the first alcoholic fermentation, and devoted to a second alcoholic fermentation) contain a majority of proteins from grape origin as compared to a relatively poor diversity of yeast proteins [16,28]. Here, we report the presence of 12 protein species from *V. vinifera* in two Champagne wines, identified as invertase and pathogenesis-related (PR) proteins, including proteins from PR-4, PR-5 and PR-14 families (Tables 1 and 2). Grape invertase and PR-5 proteins (i.e., thaumatin-like, VVTL1 and osmotin-like proteins) were the main proteins in these two types of champagne as illustrated

	Accession numbers	Protein name	Mascot score	Mr	pI	N peptides	m/z	z	Pep_delta	Ion score	Peptide sequence	Variable modification
1	tr|F6HAU0| F6HAU0_VITVI	Putative uncharacterized protein OS = *Vitis vinifera*	4128	60186	4.4	19	450.85	2	0.2398	38	TFYDQVK	
							514.85	2	0.1468	38	TFYDQVKK	
							552.77	2	−0.0047	46	TAFHFQPEK	
							557.93	2	0.244	76	GWASLQSIPR	
							573.69	2	−0.1621	45	TFFCTDLSR	
							602.72	2	−0.1484	81	SSLAVDDVDQR	
							621.82	2	−0.0602	81	KGWASLQSIPR	
							655.68	2	−0.3304	90	VYPTEAIYGAAR	
							811.55	2	0.38	78	DPTTMWVGADGNWR	Oxidation (M)
							850.56	2	0.2281	94	VLVDHSIVEGFSQGGR	
							966.89	2	−0.246	81	IYGSIVPVLDDEKPTMR	
							975.29	2	0.5643	88	IYGSIVPVLDDEKPTMR	Oxidation (M)
							977.08	2	0.1983	116	ILYGWISEGDIESDDLK	
							1010.05	2	0.0204	78	TGTYLLLWPIEEVESLR	
							1041.13	2	0.209	103	ILYGWISEGDIESDDLKK	
							1047.59	2	0.2152	90	YENNPVMVPPAGIGSDDFR	Oxidation (M)
							1054.96	2	−0.1445	62	RILYGWISEGDIESDDLK	
							746.40	3	0.0272	64	RILYGWISEGDIESDDLKK	
							1499.29	2	−0.0434	102	GALGPFGILVLADDTLSELTPIYFYIAK	
							1157.62	3	0.3184	98	HDYYALGEYDPMTDTWTPDDPELDVGIGLR	Oxidation (M)
2	tr|F6HUH1| F6HUH1_VITVI	Putative uncharacterized protein OS = *Vitis vinifera*	584	24900	5.1	8	564.80	2	0.1154	63	TSCTFDANGR	
							711.45	2	0.2618	59	APGGCNNPCTVFK	
							738.29	2	-0.1172	31	TSLFTCPSGTNYK	
							777.20	2	−0.3225	107	CTYTVWAAASPGGGR	
							953.03	2	0.1768	112	GIQCSVDINGQCPSELK	
							1009.65	2	0.2958	122	LDSGQSWTITVNPGTTNAR	
							725.48	3	0.3358	82	RLDSGQSWTITVNPGTTNAR	
							1128.06	2	0.2425	89	TNEYCCTDGPGSCGPTTYSK	

Table 1 – Proteins identified in the single-varietal Champagne by consulting the Uniprot_Viridiplantae database.

3	tr|A5C9F1|A5C9F1_VITVI	Putative uncharacterized protein OS = *Vitis vinifera*	522	24834	4.5	6	550.71	2	−0.0678	52	CPDAYSYPK
							728.46	2	0.2742	59	TTGGCNNPCTVFK
							881.30	2	−0.2569	120	GISCTADIVGECPAALK
							1220.42	2	0.8159	91	DDQTSTFTCPSGTNYEVIFCP
							1271.14	2	0.3015	110	TDEYCCNSGSCNATTYSEFFK
							1260.15	3	0.8275	81	TRCPDAYSYPKDDQTSTFTCPSGTNYEVIFCP
4	tr|F6HUG9| F6HUG9_VITVI	Putative uncharacterized protein OS = *Vitis vinifera*	488	24812	4.7	4	550.71	2	−0.0678	52	CPDAYSYPK
								2	0.2742	59	TTGGCNNPCTVFK
								2	−0.2569	120	GISCTADIVGECPAALK
								2	0.285	108	TDEYCCNSGSCSATDYSR
5	tr|E3T3S8| E3T3S8_POAPR	Putative sucrose:fructan fructosyl transferase 1 OS = Poa pratensis	195	67539	5.6	2	530.96	2	0.3829	37	AGFHFQPEK
								2	0.244	76	GWASIQSIPR
6	tr|A5BFN7| A5BFN7_VITVI	Putative uncharacterized protein OS = *Vitis vinifera*	194	13039	9.4	2	840.68	2	0.5234	134	CLSVTNTATGTQATVR
							1037.04	2	0.1682	107	YGWTAFCGPSGPTGQAACGK
7	tr|Q9LLB7|Q9LLB7_VITVI	Thaumatin-like protein OS = *Vitis vinifera*	101	25269	8.6	2	564.80	2	0.1154	63	TSCTFDANGR
							740.29	2	−0.0632	30	APDGCNNPCTVFK
8	tr|A9ZMG2| A9ZMG2_NEPAL	Thaumatin like protein OS = Nepenthes alata	82	24795	7.4	2	711.45	2	0.2618	59	APGGCNNPCTVFK
							579.48	3	0.5924	21	YFRGLCPNAYSYPK
9	tr| A0A7Z01A0A7Z0_PYRPY	Soluble acid invertase OS = Pyrus pyrifolia var. culta	81	76300	5.9	3	552.77	2	−0.0047	46	TAFHFQPEK
							655.68	2	0.6173	57	VYPTKAIYGAAR
							570.48	3	−0.5187	33	WVPDNQKIDVGIGIR
10	tr|Q05JI2|Q05JI2_CITSI	Beta-fructofuranosidase OS = Citrus sinensis	80	76522	5.8	3	552.77	2	0.9793	23	TAFHFQPQK
							655.68	2	0.6173	57	VYPTKAIYGAAR
							1157.85	3	−0.1552	33	HDYYAIGTYHEKNVTWVPDNPEIDVGIGIR
11	tr|Q850K5|Q850K5_VITVI	Non-specific lipid-transfer protein OS = *Vitis vinifera*	57	12248	9	3	455.32	2	0.1895	57	CGVSVPYK
							598.08	2	0.5875	22	ISPSTDCSKVT
							617.26	2	−0.0514	48	GGAVPAGCCSGIK
12	tr|A5AWT9| A5AWT9_VITVI	Putative uncharacterized protein OS = *Vitis vinifera*	54	25130	8.5	2	550.71	2	−0.0678	52	CPDAYSYPK
							1049.90	2	0.0055	38	TDVYCCNSGSCGPTDYSR
13	tr|Q8L897|Q8L897_PEA	Vacuolar acid invertase PsI-1 OS = Pisum sativum	48	72303	4.7	2	669.81	2	0.8704	38	VYPTRAIYGAAR

	Accession numbers	Protein name	Mascot score	Mr	pI	N peptides	m/z	z	Pep_delta	Ion score	Peptide sequence	Variable modification
1	tr|F6HAU0|F6HAU0_VITVI	Putative uncharacterized protein OS = *Vitis vinifera*	6334	60186	4.4	20	450.87	2	0.2953	47	TFYDQVK	
							514.95	2	0.3447	40	TFYDQVKK	
							552.75	2	−0.0448	44	TAFHFQPEK	
							557.83	2	0.047	72	GWASLQSIPR	
							573.75	2	−0.0227	49	TFFCTDLSR	
							602.99	2	0.3839	83	SSLAVDDVDQR	
							604.52	2	0.4348	77	LDYGKYYASK	
							622.00	2	0.3008	78	KGWASLQSIPR	
							655.79	2	−0.1094	85	VYPTEAIYGAAR	
							811.55	2	0.3808	101	DPTTMWVGADGNWR	Oxidation (M)
							850.60	2	0.316	90	VLVDHSIVEGFSQGGR	
							975.24	2	0.4754	83	IYGSIVPVLDDEKPTMR	Oxidation (M)
							977.02	2	0.0774	124	ILYGWISEGDIESDDLK	
							1010.01	2	−0.0713	89	TGTYLLLWPIEEVESLR	
							1041.04	2	0.0293	103	ILYGWISEGDIESDDLKK	
							1047.62	2	0.2811	93	YENNPVMVPPAGIGSDDFR	Oxidation (M)
							1055.23	2	0.3953	43	RILYGWISEGDIESDDLK	
							1055.38	2	0.7031	25	RILYGWISEGDIESDDLK	
							746.42	3	0.111	77	RILYGWISEGDIESDDLKK	
							1499.35	2	0.0645	102	GALGPFGILVLADDTLSELTPIYFYIAK	
							1157.53	3	0.0243	79	HDYYALGEYDPMTDTWTPDDPELDVGIGLR	Oxidation (M)
2	tr|A5C9F1|A5C9F1_VITVI	Putative uncharacterized protein OS = *Vitis vinifera*	1093	24834	4.6	9	550.61	2	−0.2529	63	CPDAYSYPK	
							643.10	2	0.6685	53	TNCNFDASGNGK	
							679.27	2	−0.0953	38	TRCPDAYSYPK	
							728.36	2	0.0743	63	TTGGCNNPCTVFK	
							881.63	2	0.3991	100	GISCTADIVGECPAALK	
							1220.14	2	0.2546	39	DDQTSTFTCPSGTNYEVIFCP	
							1271.05	2	0.125	108	TDEYCCNSGSCNATTYSEFFK	
							1260.09	3	0.6558	79	TRCPDAYSYPKDDQTSTFTCPSGTNYEVIFCP	
							1326.86	3	0.9944	114	TTGGCNNPCTVFKTDEYCCNSGSCNATTYSEFFK	
3	tr|F6HUG9|F6HUG9_VITVI	Putative uncharacterized protein OS = *Vitis vinifera*	964	24812	4.7	6	550.61	2	−0.2529	63	CPDAYSYPK	
							643.10	2	0.6685	53	TNCNFDASGNGK	
							679.27	2	−0.0953	38	TRCPDAYSYPK	
							728.36	2	0.0743	63	TTGGCNNPCTVFK	

Table 2 – Proteins identified in the blended Champagne by consulting the Uniprot_Viridiplantae database.

							881.20	2	−0.4491	110	GISCTADIVGECPAALK
							1067.04	2	0.3201	121	TDEYCCNSGSCSATDYSR
4	tr|F6HUH1| F6HUH1_VITVI	Putative uncharacterized protein OS = *Vitis vinifera*	569	24900	5	8	564.93	2	0.3736	72	TSCTFDANGR
							711.37	2	0.1101	28	APGGCNNPCTVFK
							738.30	2	−0.0923	33	TSLFTCPSGTNYK
							777.55	2	0.3677	91	CTYTVWAAASPGGGR
							953.11	2	0.3335	72	GIQCSVDINGQCPSELK
							1009.42	2	−0.1614	90	LDSGQSWTITVNPGTTNAR
							725.58	3	0.6161	64	RLDSGQSWTITVNPGTTNAR
							1128.10	2	0.3207	103	TNEYCCTDGPGSCGPTTYSK
5	tr|E3T3S8| E3T3S8_POAPR	Putative sucrose:fructan fructosyl transferase 1 OS = Poa pratensis	235	67539	5.6	2	530.84	2	0.1463	44	AGFHFQPEK
							557.83	2	0.047	72	GWASIQSIPR
6	tr|D6R2Y0| D6R2Y0_9ROSI	Pathogenesis-related protein 4 OS = Vitis hybrid cultivar	205	15674	5.5	2	840.58	2	0.3189	139	CLSVTNTATGTQATVR
							1019.70	2	0.4443	110	IVDQCSNGGLDLDSGVFNK
7	tr|E3T5W6| E3T5W6_GOSHI	Vacuolar invertase 1 OS = Gossypium hirsutum	171	72138	4.7	2	655.79	2	−0.1094	85	VYPTEAIYGAAR
							656.53	3	−0.4726	34	TVITSRVYPTEAIYGAAR
8	tr|Q850K5| Q850K5_VITVI	Non-specific lipid-transfer protein OS = *Vitis vinifera*	150	12248	8.9	3	455.41	2	0.3555	45	CGVSVPYK
							617.47	2	0.3595	64	GGAVPAGCCSGIK
							971.37	2	−0.2589	131	TFSSSVSGINYGLASGLPGK
9	tr|Q94C05| Q94C05_IPOBA	Soluble acid invertase Ib2FRUCT3 OS = Ipomoea batatas	114	72710	5	3	557.83	2	0.0572	44	GWASVQTIPR
							604.52	2	0.4348	77	IDYGKYYASK
							1151.63	3	−0.7392	41	HDYYAIGTYDPFNNTWTPDNPEIDVGIGLR
10	tr|F4HYP3| F4HYP3_ARATH	Beta-fructofuranosidase, insoluble isoenzyme CWINV3 OS = Arabid	107	65472	5.7	2	604.52	2	0.4348	77	LDYGKYYASK
							814.11	2	−0.5892	25	YYASKTFYDDVKK
11	tr|F6GXX3| F6GXX3_VITVI	Putative uncharacterized protein OS = *Vitis vinifera*	71	11023	8.9	3	455.41	2	0.3555	45	CGVSVPYK
							960.66	2	0.2993	71	TFSGSIPGINFGLASGLPGK
							960.66	3	0.4885	34	QAACKCLKTFSGSIPGINFGLASGLPGK
12	tr|A0A7Z0| A0A7Z0_PYRPY	Soluble acid invertase OS = Pyrus pyrifolia var. culta	70	76300	5.9	3	552.75	2	−0.0448	44	TAFHFQPEK
							655.79	2	0.8382	45	VYPTKAIYGAAR
							570.57	3	−0.2307	46	WVPDNQKIDVGIGIR
13	tr|Q8L897| Q8L897_PEA	Vacuolar acid invertase PsI-1 OS = Pisum sativum	67	72303	6.1	2	552.75	2	−0.0448	44	TAFHFQPEK
									0.884	54	VYPTRAIYGAAR
14	tr|Q7DLY6| Q7DLY6_ARATH	Beta-fructosidase (Fragment) OS = Arabidopsis thaliana	59	71498	5.3	3	552.74	2	−0.0656	40	TAFHFQPEK
							656.12	2	−0.4982	32	VYPTKAIYGATK
							692.90	3	−0.465	23	THKNLVQWPVEEIKSLR
15	tr|Q05JI2| Q05JI2_CITSI	Beta-fructofuranosidase OS = Citrus sinensis GN = Cs-bFruct1	51	76522	5.8	3	552.75	2	0.9392	20	TAFHFQPQK
							655.79	2	0.8382	45	VYPTKAIYGAAR
							1157.78	3	−0.3617	38	HDYYAIGTYHEKNVTWVPDNPEIDVGIGIR

Table 3 – Proteins identified in the single-varietal Champagne (100% Chardonnay) by consulting the Uniprot_*Saccharomyces cerevisiae* database.

	Accession numbers	Protein name	Mascot score	Mr	N peptides	m/z	z	pep_delta	ion score	peptide sequence	variable modification
1	sp|Q03674|PLB2_YEAST	Lysophospholipase 2 OS = *Saccharomyces cerevisiae* (strain ATCC 204508/S288c)	666	75979	7	361.59	2	−0.2004	34	DVDVFK	
						530.56	2	0.5573	32	EALHSFLSR	
						555.70	2	−0.1459	86	IGIACSGGGYR	
						658.86	2	0.0215	72	WESIVQEVQAK	
						743.36	2	−0.0195	63	NGEMPLPITVADGR	Oxidation (M)
						1250.75	2	0.2391	120	ALSYNFFPSLPDAGSALTWSSLR	
						1117.80	3	0.7525	27	GMAFPYVPDVNTFLNLGLTNKPTFFGCDAK	Oxidation (M)
2	sp|P38616|YGP1_YEAST	Protein YGP1 OS = *Saccharomyces cerevisiae* (strain ATCC 204508/S288c)	379	37419	4	453.20	2	−0.1089	59	GVLSVTSDK	
						595.32	2	−0.0333	78	NAVGAGYLSPIK	
						1041.83	2	−0.4349	86	WFFDASKPTLISSDSIIR	
						1090.49	3	0.8376	71	LVYSGVFTPPTACSYGAGLPVAIVDDQDEVK	
3	sp|P53301|CRH1_YEAST	Probable glycosidase CRH1 OS = *Saccharomyces cerevisiae* (strain ATCC 204508/S288c)	160	52840	2	541.90	2	0.2199	73	VIVTDYSTGK	
						1249.67	2	0.2613	115	YTYGDQSGSWESIEADGGSIYGR	

Table 4 – Proteins identified in the blended Champagne by consulting the Uniprot_Uniprot_*Saccharomyces cerevisiae*.

	Accession numbers	Protein name	Mascot score	Mr	N peptides	m/z	z	Pep_delta	Ion score	Peptide sequence	Variable modification
1	sp|P53301|CRH1_YEAST	Probable glycosidase CRH1 OS = *Saccharomyces cerevisiae* (strain ATCC 204508/S288c)	242	52840	4	357.89	2	0.2997	43	LEVILK	
						541.87	2	0.1553	59	VIVTDYSTGK	
						664.00	2	0.3639	56	TTWYLDGESVR	
						1249.67	2	0.2603	95	YTYGDQSGSWESIEADGGSIYGR	
2	sp|P38616|YGP1_YEAST	Protein YGP1 OS = *Saccharomyces cerevisiae* (strain ATCC 204508/S288c)	215	37419	3	595.51	2	0.3453	54	NAVGAGYLSPIK	
						694.81	3	−0.684	29	WFFDASKPTLISSDSIIR	
						1090.25	3	0.1411	56	LVYSGVFTPPTACSYGAGLPVAIVDDQDEVK	
3	sp|Q03674|PLB2_YEAST	Lysophospholipase 2 OS = *Saccharomyces cerevisiae* (strain ATCC 204508/S288c)	206	75979	4	361.82	2	0.2694	24	DVDVFK	
						555.70	2	−0.1515	85	IGIACSGGGYR	
						659.08	2	0.4772	71	WESIVQEVQAK	
						834.40	3	0.9281	60	ALSYNFFPSLPDAGSALTWSSLR	
4	sp|P28319|CWP1_YEAST	Cell wall protein CWP1 OS = *Saccharomyces cerevisiae* (strain ATCC 204508/S288c)	164	24254	3	480.05	2	−0.3929	26	SSSGFYAIK	
						699.95	2	0.2489	96	EGSESDAATGFSIK	
						974.24	2	0.5466	97	SGSDLQYLSVYSDNGTLK	

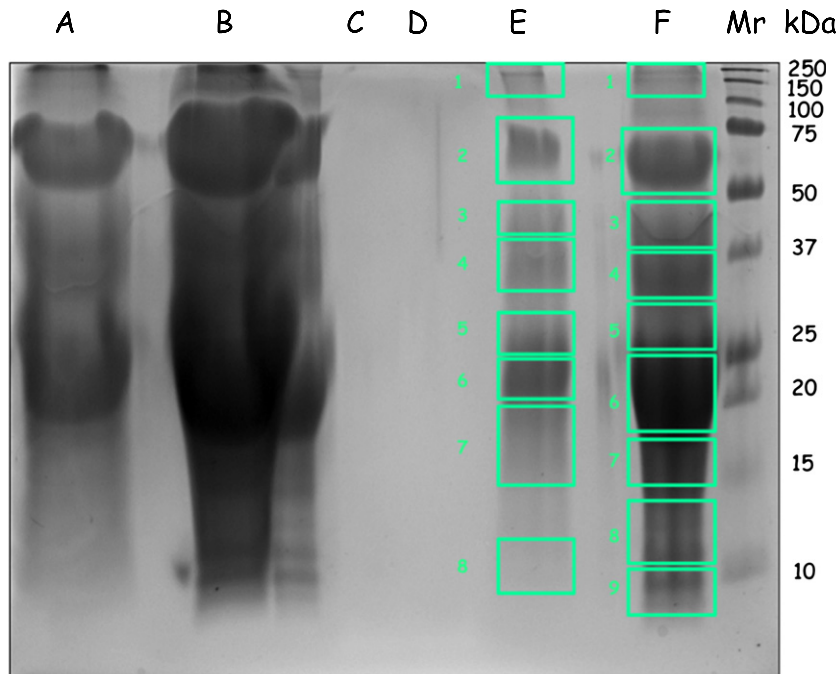

Fig. 1 – SDS-PAGE of champagne proteins captured by CPLLs. A = 80 μL 4% SDS of eluate (Champagne *Blanc de blancs*), B = 80 μL 4% SDS of eluate (Champagne *Brut*), C = 80 μL untreated Champagne *Blanc de blancs*, D = 80 μL untreated Champagne *Brut*, E = 25 μL 4% SDS of eluate (Champagne *Blanc de blancs*), F = 25 μL 4% SDS of eluate (Champagne *Brut*), and Mr = molecular mass standards. Proteins were stained with colloidal Coomassie Blue. The numbered bands were excised, digested by trypsin, and analyzed by nano-LC–MS/MS.

by the highest number of peptides retrieved for their identification. These results are in accordance with previous ones related to the proteome of Champagne base wines from the Chardonnay variety, showing a higher number of invertase and PR-5 isoforms than other grape proteins [16].

Grape invertases (encoded by GIN 1 and GIN 2) convert sucrose into glucose and fructose and GIN1 is considered as a major wine protein, regardless of grape variety [1,8,14]. This is a N-glycoprotein, which contains 642 amino acids and has a theoretical Mr of 71.5 kDa and pI of 4.6. Vacuolar invertase 1 was the main protein retrieved in both Champagne wines. This is an interesting finding since it has been previously demonstrated in a Chardonnay base wine that grape invertase is sensitive to fining treatment with bentonite, leading to a decrease in invertase content correlated to a loss of foaming properties [1]. Nevertheless, other grape proteins might be more sensitive to bentonite as demonstrated hereafter by Sauvage et al. [7]. In our study, no fining treatments were conducted on Champagne wines, except for a low amount of sodium bentonite added as a riddling agent, which thus cannot completely remove grape invertase from wine. Our result is also well correlated with the numerous invertase-identified spots in a Chardonnay base wine, which could account for different glycoforms of this protein [16]. These glycoforms might also be generated during the alcoholic fermentation, through a yeast-proteolytic activity [18].

VVTL1, a PR protein considered as a major haze protein [29], was also identified in our Champagne wines, together with other PR protein species: osmotin-like protein (PR-5 family), thaumatin-like protein (PR-5 family), PR-4 type protein and non-specific lipid-transfer proteins (PR-14 family). PR proteins are classified in 17 families and most of them have antifungal activities. Only 5 PR families have already been recovered in wine: PR-2, PR-3, PR-4, PR-5 and PR-14 families. They accumulate during fruit ripening and their expression may vary according to grape variety and environmental conditions [14,15]. Among PR proteins, non-specific lipid-transfer proteins (nsLTP) are considered as potential allergens [30] and have already been identified in still wines but never in sparkling wines [14,23,31,32]. Two members of this PR-14 family were identified in the blended champagne and one in the single-varietal champagne. nsLTPs are glycoproteins characterized by a basic pI with a low molecular mass (around 12 kDa). Other important features are their resistance to protease activity, to the alcohol content and also their stability to heat, which are likely to explain their presence in those commercial Champagne wines [30,32]. On the other hand, this class of proteins is a good foam promoter in beer [33] and thus, could be involved in champagne's foam formation.

Surprisingly, two classes of PR proteins, PR-2 (β-1,3-glucanase) and PR-3 (class IV chitinase), were not retrieved in these Champagne wines although their presence has been already demonstrated in wines from various grape varieties. Various factors related to the winemaking process are likely to explain the absence of both PR-families in champagne. Firstly, our champagne samples have undergone malolactic fermentation, in order to transform malic acid into lactic acid, before the 2nd alcoholic fermentation. In 2008, Folio et al. [34] have shown that an extracellular protease activity is secreted by *Oenococcus oeni* through the malolactic fermentation. Thus, some grape proteins, and particularly PR-3 proteins, might be hydrolyzed during malolactic fermentation or either adsorbed by lees as suggested

by Manteau et al. [25]. These authors have previously shown by SDS-PAGE analysis that a single-varietal champagne elaborated with Pinot Noir grapes was completely devoid of proteins. Indeed, both CHV5 (a chitinase isoform, PR-3 family) and TL (PR-5 family) were undetectable by SDS-PAGE coupled with immunodetection. These results were also correlated with an increase of protease activity (10 times higher than in the grape must). Indeed, yeast-secreted proteases are active toward these two classes of PR proteins [18]. In our experiment, sodium bentonite was employed as a riddling agent and thus added to the wine during the 2nd alcoholic fermentation. Bentonite acts as a cation exchanger at the wine pH and leads to a decrease in total wine protein content [1]. Low molecular mass proteins and PR-2 proteins are preferentially removed by bentonite, rather than other wine proteins [7,35], thus this might also explain the absence of PR-2 proteins in both Champagne wines.

Despite the absence of PR-3 proteins, we revealed the presence of a chitinase from the PR-4 family (D6R2Y0) in the blended champagne and (O81228) in the single varietal champagne. (*Both accession numbers lead to the same protein sequence*). Other approaches led to the identification of the same PR-4 protein (D6R2Y0 or gi|3511147) in Chardonnay, Semillon and Sauvignon blanc wines [11,16,35]. To date, seven classes of chitinases are members of four PR families (PR-3: PR-4, PR-8 and PR-11), which occur in various plant species. Two classes of chitinases (class I and class II) are represented in the PR-4 family, these proteins have a chitin-binding domain and also possess antifungal activity.

Major occurrence of glycoproteins was also observed in these Champagne wines. Indeed, the glycosylation sites of grape invertase, PR-5 proteins and yeast proteins have been confidently characterized in a Chardonnay wine by Palmisano et al. [36]. Glycosylation is a major post-translational modification (PTM) that many proteins undergo. The presence of glycans can have a significant impact on the stability, solubility, folding, proteolytic resistance and biological activity of glycoproteins. There are four types of glycosylation: (i) N-glycosylation, (ii) O-glycosylation, (iii) C-mannosylation and (iv) glycosylphosphatidylinositol (GPI) anchor. Among the diverse PR proteins identified, all have at least two putative N-glycosylation sites within their sequences, except for VVTL1 (O04708), thaumatin-like protein (Q7XAU7) and PR-4 type protein (D6R2Y0), which indicate many possible O-glycosylation sites (Ser/Thr). Another reason for the absence of PR-3 and PR-2 proteins could be linked to their glycosylation state, indeed proteolysis may be dependent on the nature of the glycans linked to these proteins, as reported by Garner et al. [37]. Thus, yeast-secreted proteases might be active toward various wine proteins. On the other hand, the presence of glycosylated proteins in Champagne wines is an important fact since they might contribute to enhance foam stability, as previously reported in beer [33]. Even if our study showed that the 100% Chardonnay Champagne wine has a lower protein content, this grape variety provides a better foam quality than other grape varieties [38]. Thus, quality might be more important than quantity, in order to determine the foaming potential of wine proteins.

Yeasts are also a source of proteins, and particularly, highly glycosylated proteins such as mannoproteins. Their presence has already been detected in a Champagne base wine, using SDS-PAGE and immunodetection [28], but, to date, the protein content of sparkling wines and champagne has been poorly investigated. This work is the first evidence of the presence of yeast proteins in a Champagne wine and in a more general way in a sparkling wine. These proteins might be released during the first or second alcoholic fermentation, or even during aging on lees. Indeed, yeast autolysis occurs after the 2nd in-bottle alcoholic fermentation of the base wine and might modify and release various nitrogen compounds (amino acids, peptides, proteins) in the wine as reviewed by Alexandre and Guilloux-Benatier [24]. The yeast mannoproteins were found to improve the stability of wine against protein haze, and to increase the foaming properties of sparkling wines. Indeed, some strains of yeast have the capacity to improve the foaming properties of sparkling wine [39]; but it is difficult to know if these strains release foam-active yeast proteins or if they degrade less foam-active grape proteins. In addition, the proteomes of other sparkling wines (Cava) have been investigated through other proteomic approaches less sensitive than CPLL, and the presence of yeast proteins was highly suspected, due to the presence of high molecular mass glycoproteins, but not identified [40]. A recent study has identified, by SDS-PAGE and LC–MS/MS, the proteins released by yeast in a model wine [41]. They revealed a wide diversity of secreted yeast proteins, and most of them were also identified in our samples. Indeed, 7 yeast proteins were identified in these Champagne wines (Tables 3 and 4) and 4 of them (Q03674, P38616, P53301, P28319) have already been retrieved in still red or white wines [12,14,21,36], or even model wines [41,42], whatever the grape variety or the winemaking process. All these yeast proteins are ranging from 24 to 137 kDa and have an acidic pI, except an uncharacterized protein YEL025C (P39991) with a basic pI value (Supplementary Table 3). Our experiment does not allow us to distinguish whether they have been released during the first or the second alcoholic fermentation. However, one yeast protein, CWP1 (P28319), has already been identified in a Champagne base wine [16] and thus, could be released in the wine during the first alcoholic fermentation.

4. Conclusions

It is important to point out that our study reports, for the first time, the proteome of a Champagne wine, though no differentiation according to grape varieties can be done at this stage. Thanks to CPLLs, a wide variety of proteins has been identified and belongs to various classes of proteins from grape and yeast. Among all these identified proteins, some of them might be good candidates to enhance the foaming properties of Champagne and sparkling wines as well, according to their glycosylation nature or to previous studies related to their foaming potential. Recently, Vincenzi et al. [40] suggested that high molecular mass glycoproteins (likely mannoproteins) and PR-proteins interact to promote foamability of Prosecco, an Italian white sparkling wine elaborated with Glera grapes.

Our work provides a thorough list of the proteins likely to be found in two standard commercial Champagne wines and, thus, likely to impact their quality. However, we must also keep in mind that this proteome is not static and might change according to various parameters such as blending, fining, or aging. In the Champagne making process, the

blending of various base wines from different grape varieties, *terroir* and vintage is a crucial and key step, which will result in distinct Champagne wines after the *prise de mousse*. Further studies related to the proteome of old Champagne wines would also be interesting in order to evaluate precisely, and in real conditions (not in a model wine), the influence of aging on lees.

Actually, the autolysis of yeast occurs during the aging of champagne on lees. During this process, the yeast releases various compounds which progressively modify the organoleptic properties of wine, providing them their roundness and characteristic aroma and flavor, as detailed in the review by Alexandre and Guilloux-Benatier [24]. Moreover, this aging period is also required to produce smaller bubbles, simply because the wine progressively loses a bit of dissolved CO_2 through the cork stoppers or caps used to close the bottles [43,44].

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Appendix A. Supplementary data

Supplementary data to this article can be found online.

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