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Exploring putative kokumi oligopeptides in classic sparkling wines with a UHPLC-ESI-MS/MS targeted protocol

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ABSTRACT

This study started from the observation that some oligopeptides are capable of imparting kokumi properties to various solid and liquid foods, but to date this topic has not been addressed in wine. A targeted metabolomics method using UHPLC-MS/MS capable of quantifying both amino acids and oligopeptides in wines was therefore developed and validated, confirming the presence of 50 oligopeptides in wine, most of which were previously unexplored. In-silico screening of the affinity of these oligopeptides to interaction with the CaSR protein, necessary to activate kokumi sensations, allowed us to identify 8 di-peptides and 3 tri-peptides that are putative kokumi compounds. The presence of these compounds was explored in a representative sample of Trentodoc classic method vintage sparkling wines, highlighting that they are ubiquitous, with concentrations and profiles that are very reproducible even in vintages from the same cru, and with significant variability between wines from different wineries. The average concentration of kokumi oligopeptides was 19.8 mg/L, in a range between 9.1 and 33.3 mg/L. Half of the sparkling wines analysed also contained glutamic acid at concentrations equal to or greater than the threshold for the umami property, namely 48 mg/L. Sensory tests on the dipeptide Gly-Val confirmed the ability of this novel kokumi compound to modify the taste of wines in a complex, not easily recognisable but statistically significant way, but not that of a model wine solution. Preliminary laboratory-scale fermentation tests allowed us to observe that the oligopeptide profile in wines is linked to the starting grape matrix, and is completely different from that obtainable by fermenting barley or apple juice with the same yeast.

Keywords

LC-MS/MS, wine; sparkling wine; oligopeptides; kokumi; glutamic acid; umami; beer

1. INTRODUCTION

The term "kokumi" has been strongly associated with oligopeptides in food science. It refers to a complex flavour, characterised by thickness, mouthfulness and continuity. The isolation and characterisation of sulphur-containing compounds, including glutathione (GSH), which are capable of enhancing taste in an umami solution, even when added even at subthreshold level, was first characterised by Ueda et al working on garlic and onion [1,2]. A group of three γ-glutamyl small peptides, corresponding to γ-L-glutamyl-L-leucine, γ-L-glutamyl-L-valine, and γ-L-glutamyl-L-cysteinyl-β-alanine were demonstrated to act as the key contributors to the kokumi taste of thermally treated beans (*Phaseolus vulgaris L.*). The synergistic effect of these compounds is striking. A clear example of this was the threshold of γ-glutamyl-cysteinyl-β-alanine, which decreased by a factor of 32 (from 3.8 down to 0.1 mmol/L) in a binary mixture of glutamic acid and sodium chloride compared to water [3]. The list of bioactive oligopeptides was further enriched by the discovery of another series of γ-L-glutamyl peptides, in particular γ-Glu-Glu, γ-Glu-Gly, γ-Glu-Gln, γ-Glu-Met, γ-Glu-Leu, and γ-Glu-His, identified for the first time as the key kokumi molecules enhancing mouthfulness and taste continuity in mature Gouda cheese, where they were present in concentrations between 4.1 and 17.7 μmol/kg [4]. The main pathways for the generation of taste-active peptides in food fermentation have been reviewed [5]. Proteolysis or autolysis during food fermentation generates taste-active amino acids and peptides in soy sauce, cheese, fermented meats, and bread. However, our understanding of microbial metabolic activities related to the formation of taste-active peptide derivatives is still incomplete [5].

To summarise, it is now accepted that the kokumi sensation is important for determining the taste, flavour, mouthfeel and aftertaste quality of a wide variety of foods, but has never been explored in wine. This study aims therefore to address an unexplored question: is the “kokumi” effect significant for wine? More precisely, this study was aimed at screening for the presence of oligopeptides as kokumi taste-related substances in wine. A significant number of oligopeptides have so far been identified in fermented food products and yeast extracts. Molecules with kokumi

properties are able to enhance in particular the properties of umami substances, with which they have been closely associated [6]. Both umami and kokumi sensory descriptors are not easily recognisable in natural ingredients compared to other basic tastes, and to date they have not been sufficiently explored in fermented beverages such as beers and wines. As already highlighted by Peter Klosse [7], the positive effects of umami on flavour are well documented, but wines are not mentioned in the literature on umami. Likewise, in scientific publications on wine, references to umami are scarce, if not absent. Recent studies on the free amino acid content of fermented beverages [8] have suggested that beverages having long contact with yeast may contain higher amounts of free glutamate and therefore have a greater capacity to impart umami than beverages with little or no contact with yeast, and that the copresence of umami substances could provide an explanation for some food pairings capable of eliciting a synergistic umami taste, as in the case of champagne and oysters [9]. Another recent study aimed at evaluating the effect of amino acids and their interaction with volatile and other non-volatile substances on the mouthfeel properties of red wines concluded that the source, presence and modulation of L-glutamic acid/glutamate in wine are virtually unexplored in wine science [10].

The umami taste threshold was identified by a trained panel in a neutral white wine with added monosodium glutamate (MSG), showing that 50% of the panellists recognised this taste at the MSG concentration of 48 mg/L [11]. The authors commented that this recognition of the umami taste in wine, with a much lower taste threshold than in water, could be due to the presence of other umami-related molecules responsible for taste perception, or acting in synergy, thus promoting its recognition. This is an important observation, considering that concentrations of glutamic acid above 48 mg/L are frequently observed in wines [10].

A recent study has proposed a model to understand the mechanism of interaction occurring when glutamate is present in complex food matrices, where it is suggested to facilitate the binding of kokumi receptors to compounds present in the matrix [6]. In turn, these bound kokumi substances increase the intensity of umami, sweet, salty and fatty tastes, with a consequent increase in

palatability accompanied by kokumi flavour, with descriptors such as thickness, fullness and continuity. This is therefore a particularly difficult problem to address from a sensory point of view, being much more complex than the investigation of a single, specific sensory descriptor and linked to the presence of a single class of molecules that induce it. This complexity, common to food matrices, must be considered to explore the possibility that it is present in wine. Among known kokumi substances, the tripeptide glutathione is present in wine. Unfortunately, reduced glutathione (GSH), which is present in very significant concentrations in grapes, is usually found at the level of a few mg/L in wines, and its content decreases over time, being involved in various oxidative reactions. Favoured by the presence of oxygen, GSH in wine gives glutathione disulphide (GSSG) which reacts with SO3H- to provide glutathione S-sulphonate (GSSO3H) [12].

The most powerful kokumi substance is the tripeptide γ-glutamyl-valyl-glycine (γ-Glu-Val-Gly). In controlled studies, this particular peptide intensified savoury, salt, and sweet flavours at least 10 times more than glutathione, and it is highly stable. The results show that γ-Glu-Val-Gly is widely distributed in fermented foods such as fish sauces, soy sauce, fermented shrimp paste, and beer. This peptide was present at sub mg/L level in beers, but was not detected in any of the wine or rice wine samples [13].

Given the surprising lack of methods aimed at exploring oligopeptides in wines, we began the study by developing and validating a metabolite profiling method that would allow the exploration of amino acids and a large number of oligopeptides in wines with a single analysis, through separation using high-performance liquid chromatography, combined with detection by a tandem mass spectrometer. The novel LC-MS protocol was then applied to a survey on white wines. We chose to start our survey with samples of classic sparkling wines. The evolution of the mouthfeel of classic sparkling wine during refinement in the bottle, which can last many years, in anoxia and in contact with yeasts, is an intriguing process and is still not entirely understood. In this study we wanted to explore the composition of oligopeptides, and especially low molecular weight peptides in wines, with particular attention for sparkling wines with the Italian Trentodoc denomination.

However, the study did not wish to limit itself to identification and quantification of the oligopeptides present, but also to make use of a validated strategy suitable for selecting the compounds capable of eliciting the kokumi sensation, among those identified. From a mechanistic perspective, the ability of some molecules to elicit kokumi perception has been associated with their ability to interact with the calcium sensing receptor (CaSR), resulting in its allosteric activation [14]. CaSR is a Class C G-protein coupled receptor (GPCR) responsible for maintaining Ca^{2+} homeostasis in blood. Its primary sequence has 1078 amino acids, and it is mainly expressed in the parathyroid glands, thyroid and kidneys [15]. However, its involvement in kokumi sensing is related to the broader expression of this receptor, which has been described in the gustatory tissues of rats and taste buds in the lingual epithelium [16]. More specifically, the part of the protein involved in interaction with kokumi-active substances is the so-called Venus Fly Trap (VFT) domain (**Figure 1A**). In agreement with the mechanistic model previously described, this region can close over ligands and this event is reported as the initiating event underpinning CaSR activation and kokumi perception [17].

Precise knowledge of the mechanism of action of kokumi oligopeptides therefore allowed us to test in-silico the affinity of di- and tri-peptides identified in wines with the CaSR protein, to select potentially active compounds. Computational screening looking for oligopeptides potentially active for kokumi among the library of those identified in wine was performed, following the approach reported by Dellafiora et al [18]. In order to provide fit-for-purpose procedural validation, the two dipeptides Ala-Val and Leu-Ala were included in the study as negative and positive controls [19], respectively. This in-silico approach also has the potential to identify new potential kokumi tasting sequences.

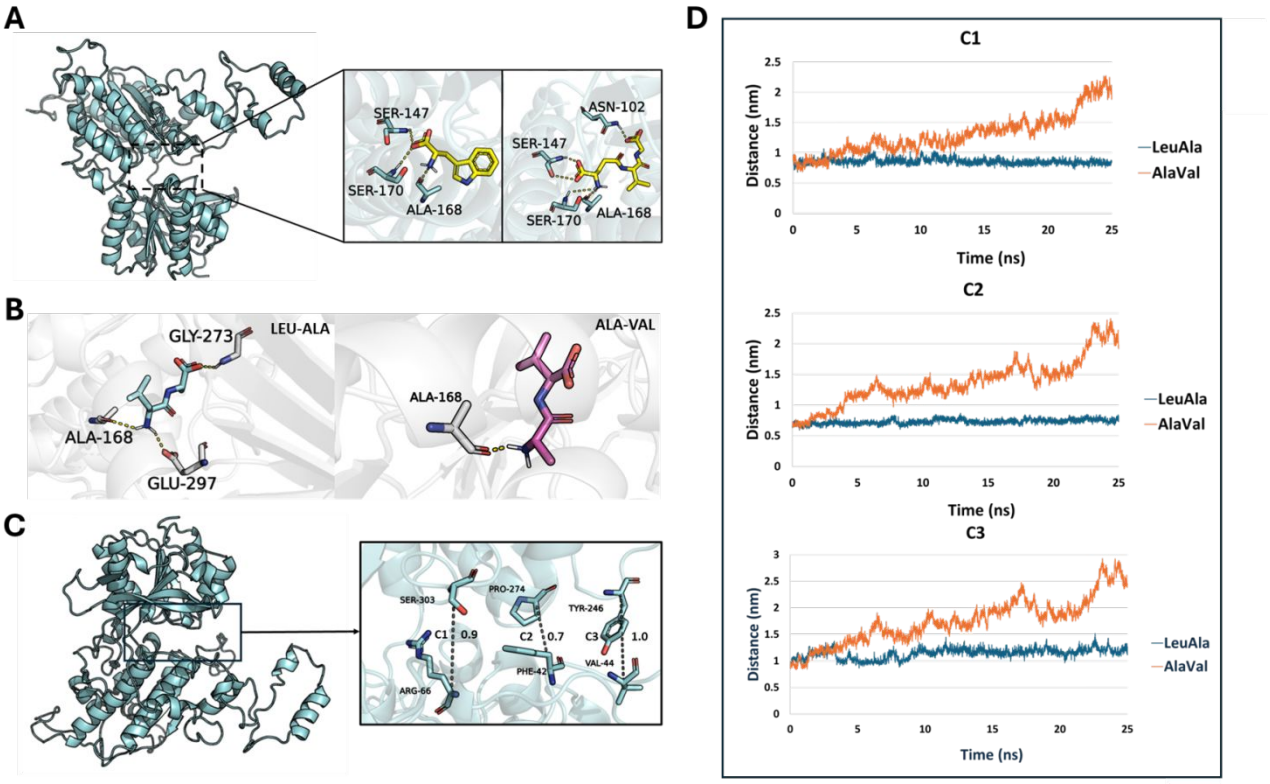

Figure 1. Graphical representation of CaSR VFT domain and molecular modelling results for the model validation. The protein is represented in cartoon, ligands and amino acids involved in polar interactions are represented in sticks, and polar interactions are represented as yellow dashed lines. **A**. The CaSR VFT domain is represented in cartoon, while in the close-up on the right the interaction with two kokumi active compounds (L-Trp on the left and γ-Glu-Val-Gly on the right) from a previous work [18]. **B.** Binding pose of the positive and negative controls Leu-Ala and Ala-Val, respectively. **C.** The VFT domain residues involved in the ligand-dependent closure are shown. The dashed lines indicate the distances (nm) between the alpha-carbons of C1, C2 and C3 monitored in MD. The distances reported are those of the crystal structure with PDB code 5FBK. **D.** Interatomic distances (nm) of C1, C2 and C3 over time for Leu-Ala and Ala-Val.

It remained to explore whether the identified bioactive compounds could actually have a kokumi effect in wine. While waiting to conduct more in-depth sensory studies, we started with the exploration of a single dipeptide, Gly-Val, selected from the complex pool of putative kokumi

peptides detected in wine, and present in wines but never studied in literature on kokumi properties. Gly-Val was explored using sensory analysis. Triangle tests (TT) followed by 3-alternative forced choice (3-AFC) were performed to estimate the oral detection threshold of Gly-Val in model and real white wines. Moreover, the potential kokumi activity of Gly-Val on white wine taste and mouthfeel sensations was explored with descriptive analysis with relative to reference scaling (DA with RR scaling).

Last but not least, we wondered whether the compounds observed in wines could be produced directly by yeasts, or whether they were degradation metabolites of the grape protein fraction, and therefore specific to the wine matrix. In this case, we again conducted preliminary simple fermentation tests of must and other fermentable matrices on a laboratory scale, to verify the specificity of the pattern of wine oligopeptides compared to other fermentable matrices (apple and barley).

1. MATERIALS AND METHODS

2.1 LC-MS analysis

2.1.1 Chemicals. The majority of the chemical standards are commercially available and were obtained from different suppliers (**Table S1**). The wine, a light red and a neutral white for preparation of the curve, was Tavernello (Caviro, Italy)

2.1.2 Preparation of standard solutions. The choice of metabolites was mainly based on a literature search, based on their reported presence and/or potential relevance for wine quality, and potential association with descriptors such as sapidity and savouriness. Stock solutions of each individual standard were prepared in pure methanol with 20% water and all stock solutions were stored at −20 C. Glutathione standard aliquots are best stored at - 80 °C. Glutathione powder is stable at 4 °C for a few years, but it oxidises rapidly once in aqueous solution. These stock solutions were used to prepare 3 standard mixtures including circa one third of the compounds each. Working calibration curves were obtained by dilution in methanol/water 1:1 to obtain 16 calibration points

(dilution factors of 1−20000) for linear dynamic range assessment. The composition of each mixture and the starting concentrations of the analytes are reported in **Table S1**.

2.1.3 Sample preparation. The wine (red and white table wine) was analysed after dilution in methanol/water 1:1 and filtration through 0.2 μm PTFE filters.

2.1.4 Liquid chromatography. UHPLC-ESI-MS/MS analysis was conducted on an AB Sciex 6500+ triple quadrupole coupled to a Shimadzu LC-30 AD pump (AB Sciex, Milan, Italy). Chromatographic separation was performed on a Luna Omega 2.1 × 150 mm Polar C18, 1.6 μm particle size (Phenomenex, California, USA). Mobile phase A was water containing 0.1% formic acid; mobile phase B was acetonitrile containing 0.1% formic acid. The flow was 0.35 mL/min, with a multistep gradient profile: 0 to 1.0 min, isocratic 1% B; from 1.0 to 3.5 min, linear gradient to 20% B; from 3.5 to 5 min, isocratic 20% B; from 5 to 5.8 min, linear gradient to 80% B; from 5.8 to 7.0 min, linear gradient to 94% B; from 7.01 to 9.0 min, wash at 100% B; from 9.01 to 12 min, back to the initial conditions. The injection volume of both the standard solutions and the samples was 2 μL. Total analysis time was 12 min. The column oven was set at 40 °C. R0 solvent was water 0.1% FA, and R3 solvent was 2-propanol. ESI was operated in positive- and negative-ion modes. Two multiple reaction monitoring (MRM) transitions were set for each metabolite, with a target cycle time across the MRM experiment set at 0.1 s. Curtain gas (CUR, air) was set at 35 PSI, and ion source gas (IS, air) 1 and 2 were set at 45 and 55 PSI respectively. The collision gas was nitrogen. The source temperature was 400 °C; ion spray voltage was 5500 V in positive-ion mode and 4500 V in negative-ion mode. Compound-dependent parameters declustering potential (DP), entrance potential (EP), collision energy (CE), and collision-cell exit potential (CXP) were determined by direct infusion of the pure standard. The MS signal was acquired only in the analyte elution window (from 0 to 7.5 min). Data were processed using MultiQuant 3.0 software (AB Sciex, Milan, Italy). Statistical analysis was performed with Statistica v.13.3 (TIBCO Software Inc.,

206 Palo Alto, CA, USA) and Metaboanalyst 5.0. A list of all the metabolites, their PubChem CID,
207 retention time, and optimised MS conditions is given in **Table 1**.

208 **Table 1**. List of all analytes, their PubChem accession identifier for a unique chemical structure
209 (CID), retention times (min), selection of the mass fragments in Q1 and Q2 for selective detection
210 in multiple reaction monitoring (MRM), ESI+, and optimised MS conditions (Volts).

211

NAME	CID	RT	Q1Mass	Q2Mass	DP	EP	CE	CXP
	Pubchem	(min)	(Da)	(Da)	(v)	(v)	(v)	(v)
Glycine	750	1,00	76,10	30	30	7	9	14
				47,9	30	7	19	14
L -Histidine	6274	0,96	156,10	110	60	3	19	12
				93	60	3	29	12
L-Alanine	5950	1,05	90,05	44,05	20	3	12	15
				42	20	3	12	15
L-Arginine	6322	0,99	175,40	70	80	11	38	8
				60,1	80	11	24	10
L-Asparagine	6267	1.02	133,20	74,1	10	7	13	15
				70	10	7	13	15
L-Aspartic acid	5960	1,03	134,10	74	7	15	25	10
				88,1	7	15	19	10
L-citrulline	9750	1,12	176,30	70,2	80	4	12	40
				86,1	80	4	9	33
L-cysteine	5862	1,13	122,03	58,99	15	6	17	15
				76,02	15	6	30	15
L-glutamic acid	33032	1,09	148,20	84,1	15	3	21	10
				56,1	15	3	36	10
L-glutamine	5961	1,06	147,08	130,2	20	3	12	15
				84,05	20	3	22	15
L-isoleucine	6306	2,95	132,10	86,2	30	15	14	10
				69,1	30	15	23	10
L-Leucine	6106	3,15	132,10	86,1	35	15	14	15
				44,2	35	15	33	15
L-Lysine	5962	0.9	147,20	84.08	15	14	14	10
				130,08	15	14	25	10
L-methionine	6137	1,91	150,30	133,2	20	15	12	15

				104	20	15	14	15
L-Phenylalanine	6140	4,41	166,20	103	35	10	36	15
				77	35	10	49	15
L-proline	14542	1,24	115,90	70,1	55	14	25	10
				68,1	55	14	30	10
L-pyroglutamic acid	7405	2,59	130,30	84,1	20	14	18	10
				56,1	20	14	33	10
L-serine	5951	1,02	106,30	60,1	25	9	11	16
				88,1	25	9	15	16
L-Threonine	6288	1,06	119,80	74,1	40	9	11	10
				102	40	9	15	10
L-tryptophan	6305	4,85	205,20	188,1	25	10	13	25
				146	25	10	24	25
L-Tyrosine	6057	3,37	182,10	136	25	15	35	15
				91	25	15	18	15
L-Valine	6287	1,58	118,10	72,1	25	10	15	15
				55	25	10	25	15
Thr - Phe	7010580	5,01	267,10	166,1	20	6	18	10
				120,2	20	6	31	10
Gly - Val	2724807	2,74	174,90	129,1	30	6	14	30
				72,1	30	6	25	30
Val - Gly	6993111	2,31	175,10	72,1	95	10	15	30
				55,1	95	10	40	25
Leu-Ala	81721	4,48	203,30	86,2	100	12	15	20
				157,3	100	12	18	20
Ala-Tyr	92946	4,55	253,40	182,3	40	10	16	18
				136,2	40	10	28	18
Tyr-Ala	5496455	4,47	253,30	136,3	70	10	18	12
				91,1	70	10	51	20
Phe-Ala	5488196	4,70	237,30	120,2	100	8	32	20
				103	100	8	52	20
Ala-Leu	96801	4,72	203,30	132,3	100	10	15	20
				86,3	100	10	25	20
Gly-Gly	11163	1,22	205,30	170,1	110	13	15	25
				84,1	110	13	36	25
Ala-Phe	96814	5,00	237,20	166,1	90	4	13	20
				120,1	90	4	30	20
Asp-Gly	151148	1,08	191,10	110	50	6	15	20
				156,2	50	6	24	20
Asp-Val	7009616	2,93	233,30	187,2	20	6	15	30
				72,1	20	6	28	30
Asp-Met	21488123	3,75	265,40	150,2	30	9	18	20
				133,1	30	9	27	20

Asp-Tyr	152455	4,57	297,30	136,2	35	12	33	20
				90,9	35	12	65	20
Asp-Leu	6992367	4,74	247,40	201,2	20	6	16	20
				86,1	20	6	27	20
Asp-Ile	21845165	4,58	247,40	201,1	30	5	16	20
				86	30	5	25	20
Ala-Asp	99719	1,12	205,30	134,1	30	5	15	20
				88,1	30	5	25	20
Gly-Hval	n.a.	1,16	191,20	173,2	20	5	12	10
				70,1	20	5	28	10
HVal-Gly	n.a.	1,25	191,20	173,2	20	5	12	10
				70,1	20	5	28	10
HPro-Pro	6156945	3,24	229,40	116,2	35	5	22	10
				86	35	5	25	10
Phe-Thr	10445608	4,56	267,30	120,2	20	8	22	10
				103,2	20	8	60	10
Asp-Asn-Val	145454376	4,11	347,20	230,1	25	8	22	10
				284,3	25	8	17	10
Asn-Asp-Val	145454062	4,47	347,20	230,1	25	8	22	10
				284,3	25	8	17	10
Asp-Asp-Val	23653186	4,57	348,10	231,2	30	10	15	20
				184,8	30	10	27	20
Asp-Gly-Val	101198875	4,53	290,30	226,3	35	11	18	20
				71,9	35	11	40	20
Asp-Gly-Leu	9901041	4,92	304,40	132,2	30	8	20	23
				86	30	8	30	23
Asp-Gly-Ile	23650515	4,78	304,40	132,2	30	8	20	23
				86	30	8	30	23
Leu-Ser-Phe	14389381	6,79	366,20	201,2	35	8	17	20
				120,1	35	8	41	20
Phe-Ser-Phe	145457315	6,82	400,40	235	35	4	17	20
				120,1	35	4	48	20
Phe-Thr-Phe	70405553	6,78	414,40	249,2	20	8	17	30
				120,1	20	8	54	30
Gly-Val-Gly	11665805	3,11	232,40	129,2	35	11	12	20
				157,3	35	11	20	20
Val-Gly-Gly	7004847	2,22	232,40	157,3	35	11	12	20
				129,2	35	11	20	20
Pro-Gly-Hpro	n.a.	1,09	267,30	220,9	40	10	20	10
				99	40	10	37	10
L glutathione	124886	2,10	308,10	179	20	15	30	10
				290,1	20	15	19	15
L glutathione ox	65359	4,23	613,10	355,1	35	15	33	14

				484,2	35	15	25	12
trans-4-hydroxy L-prolin	440575	1,05	132,20	68	20	11	30	30
				86,2	27	11	33	30
trans-4-hydroxy L-isoleucine	6918732	1,62	148,10	74,1	20	12	19	20
				84,3	20	12	17	20
L-Norvaline	65098	1,56	117,90	72,1	42	10	10	15
				54,9	40	10	33	15
L-ornithine dihydrochloride	80341	0,90	133,10	70,1	25	10	14	15
				116,1	25	10	27	15
Tyr-Phe	3421919	5,70	328,90	136,1	30	12	22	20
				90,9	30	12	60	20
Pro-Thr	18218237	1,45	217,10	70,1	40	12	20	20
				68	46	12	60	20
Cyclo Pro-Thr	11937742	4,26	199,10	125,1	30	12	21	20
				70	35	12	36	20
Cyclo Gly-Pro	126154	4,32	155,10	70	45	12	29	20
				82	45	12	28	20
Gly-His	7023107	0,94	213,10	110	20	12	30	20
				83	20	12	50	20
Leu-Gly	97364	4,20	189,20	86,1	20	12	15	20
				69,1	20	12	34	20
Pro-Leu	444109	4,71	229,10	70	35	12	20	20
				68,1	40	12	30	20
Thr-Pro	9834371	3,03	217,10	116	20	12	20	20
				70	30	12	41	20
Met-Pro	11893572	4,38	246,90	116,1	30	12	18	20
				70	30	12	50	20
Glu-Glu	439500	1,39	276,90	84,1	30	12	27	20
				130	20	12	38	20
Leu-Leu-Leu	82546	6,76	358,10	86,1	25	12	21	20
				132,1	40	12	30	20
Leu-Leu	76807	5,64	245,10	86	30	12	37	20
				69	20	12	43	20
Cyclo Met-Pro	6428989	5.80	229,00	181,1	20	12	20	20
				125,2	15	12	36	20
Ala-Gly	6998029	1,08	146,90	56	15	12	22	20
				83	15	12	55	20
Pro-Met	7408173	4,32	246,90	70	30	12	40	20
				68,1	33	12	65	20
Leu-Pro	80817	4,74	229,10	116,2	25	12	19	20
				86,1	27	12	21	20
Ile-Pro-Ile	94701	6,32	342,10	229,3	30	12	25	20
				70	40	12	67	20

Asp-Phe	93078	4,81	280,90	120,1	30	12	30	20
				103	30	12	56	20
Gly-Asp	97363	1,05	190,80	88	24	12	24	20
				74	15	12	33	20
Ala-Pro	83525	4,47	169,10	70	23	12	24	20
				98	44	12	24	20
Ala-Lys	7016106	0,90	218,20	84	40	12	20	20
				130,2	40	12	31	20
Gly-Gln	123913	1,06	204,00	130,1	20	12	22	20
				84	15	12	36	20
y-Glu-Cys	10171468	1,98	251,10	84	25	12	27	20
				142	30	12	30	20
Gly-Pro-Glu	3080768	2,54	301,90	70,1	20	12	26	20
				127	30	12	55	20
Arg-Gly-Asp-Ser	107775	1,06	434,30	169,1	70	12	38	20
				330,1	70	12	44	20
N-Acetyl-Asp-Glu	188803	3,89	304,90	84	20	12	37	20
				88	20	12	41	20
Gln - Val - Gly	124155756	3,53	302,90	182,1	30	12	27	20
				84	30	12	50	20
Glu-Val-Gly	121513936	3,49	303,90	72,1	30	12	52	20
				84	35	12	35	20
Gln - Val - Gly - Met - Glu	n.a.	4,62	563,50	182,2	60	12	34	20
				200	75	12	43	20
Glu - Cys - Met - Gln	n.a.	4,26	510,30	130,2	62	12	25	20
				147,2	50	12	33	20
Gln - Val - Gly - Met	n.a.	4,71	434,20	183,2	50	12	36	20
				138,2	50	12	60	20
Gly-Glu	99278	1,19	205,10	84	20	12	21	20
				130,2	20	12	33	20
Trp-d5 std	12209747	5,02	210,30	192,1	25	12	14	15
				150	25	12	25	15

2.1.5 Wine samples. Two sets of commercial wines were selected for initial investigation. Trentodoc vintage classic sparkling wines (Trento, Italy) were selected for their long ageing on yeast. Five vintages (2017-2021) of Ferrari Perlè Trentodoc vintage Blanc de Blanc were selected for initial exploratory analysis due to their particular taste and great persistence. Samples were obtained in triplicate during the period of maturation on the lees in cellars before disgorgement. A

total of 15 bottles were sampled and immediately analysed. Another survey on the presence of the 11 putative kokumi peptides, glutamic acid and glutathione was performed on a selection of 34 commercial vintage Trentodoc sparkling wines, sampled in April 2024, and belonging to vintages from 2011 to 2019. These wines were also analysed immediately after sampling.

2.2 Molecular modelling

2.2.1 Model construction. The crystallographic structure of the human CaSR receptor used in this work was derived from a previous validated model obtained through the trRosetta web server (https://yanglab.nankai.edu.cn/trRosetta)[20] using the FASTA sequence of the Protein Data Bank (PDB) structure 5FBK as input[21]. In agreement with a previous study[18], the VFT domain (residues 22-539) was modelled, being involved in the interaction with kokumi-active substances [22]. From a mechanistic point of view, a previous study[18] pointed out the importance of three intramolecular contact regions (C1, C2 and C3; **Figure 1C**) defined between the α-carbon of Arg66-Ser303, Pro274-Phe42 and Tyr246-Val44 in estimating the kokumi taste of molecules. The ability of the molecules analysed in this study to act on those distances was therefore investigated, to predict their possible kokumi taste. 3D structures of the whole set of peptides analysed in this study were built in .mol2 format using the PyMol building tool (version 2.4.0), setting the C- and the N-terminal as deprotonated and protonated respectively. The two Ala-Val and Leu-Ala sequences were reported as the inactive sequence and strong kokumi active sequence, respectively [19]. For this reason, they were included in the study as negative and positive L-peptide controls, along with γ-Glu-Val-Gly, the strongest kokumi active peptide identified *in vitro* to date [23].

2.2.2 Molecular docking. Docking analysis using GOLD software (Genetic Optimization for Ligand Docking, version 2022.4) was performed, as it has already succeeded in providing reliable binding architectures for proteins and peptides[18]. The binding site was defined within a 10 Å radius sphere around the centroid of the co-crystallised ligand of 5FBK [22]. 10 poses for each ligand were generated and the internal scoring function PLPScore was used, as it has been optimised for prediction of ligand

244 binding positions (the higher the score, the higher the expected peptide-pocket fitting, according to
245 the manufacturer's declaration https://www.ccdc.cam.ac.uk). Docking was assisted by "constraint
246 similarity" (weight score of 50 units), choosing the pose of the co-crystallised ligand L-Trp reported
247 in the PDB structure with the code 7DTU [24] as template and choosing to avoid the generation of
248 docking results when constraint is physically impossible.

249 **2.2.3 Molecular dynamics.** Molecular dynamics (MD) simulations were performed to assess the
250 stability of the VFT domain when in complex with selected di- and tri-peptides from sparkling wine.
251 The best scored docking pose was used as input for each complex. MD simulations were performed
252 using GROMACS (version 2019.4) [25] with CHARMM27 all-atom parameters force field parameters
253 support [26]. The complexes were solvated with SPCE waters in a dodecahedron periodic boundary
254 condition and the net charge was neutralised by adding Na^+ and Cl^- as counter ions. Then the system
255 was energetically minimised to avoid steric clashes and correct improper geometries using the
256 steepest descent algorithm with a maximum of 5000 steps. All the systems then underwent isothermal
257 (300 K, coupling time 2 psec) and isobaric (1 bar, coupling time 2 psec) 100 psec simulations before
258 running 25 nanosecond simulations (300 K with a coupling time of 0.1 psec and 1 bar with a coupling
259 time of 2.0 psec).

260

261 **2.3 Sensory analysis**

262 **2.3.1 Chemicals and materials.** Food-grade chemicals: tartaric acid, sucrose, caffeine, sodium
263 chloride (NaCl), and tannic acid were purchased from ACEF (Piacenza, Italy); monosodium
264 glutamate (MSG) and glutathione (GSH) were purchased online from Ajinomoto (Tokyo, Japan),
265 and DermoLife (Trento, Italy), respectively; ethanol (EtOH) came from VWR (Milan, Italy).

266 Sulphuric acid (H2SO4) and sodium hydroxide (NaOH) were purchased from VWR (Milan, Italy).
267 L-Leucyl-l-Alanine (Leu-Ala) and Glycyl-l-Valine (Gly-Val) were synthesised by Chemspace LLC
268 (Monmouth Junction, NJ, USA) with purity higher than 98%. Toxicity predictions obtained with

ToxibPred (an online bioinformatics tool, http://crdd.osdd.net/raghava/toxinpred/) indicated that none of peptides posed a safety risk and could be used for sensory evaluation.

2.3.2 Sensory panel. Thirty-two panellists (22–53 years old; 15 females, 17 males) were recruited among students and researchers at the University of Naples Federico II (Department of Agricultural Sciences, Division of Vine and Wine Sciences), and selected based on their interest, availability, and sensory abilities. They were all expert wine tasters with previous experience in performing descriptive sensory analysis tests. All procedures were conducted in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments, or comparable ethical standards. The Code of Ethics was approved by the Ethics Committee for Non-Biomedical Human Research (CERSUB) of the University of Naples Federico II (PG/2024/0037120). Participation was on a voluntary basis, and prior to the experiments, tasters were required to sign an informed consent form disclosing the type of research, voluntary participation and agreement to taste and spit reference solutions and wines. All data were collected anonymously.

2.3.3 Procedure. For each sample, in all sessions, 40 mL were served in covered clear ISO glasses[27] coded with three-digits, served at room temperature (21±1°C) and evaluated in individual booths[28]. Panellists were instructed to rinse their mouth with bottled still water between each triad of samples/sample.

2.3.4 Training sessions. All panellists had prior experience in the sensory evaluation of taste-active compounds in wine and could identify the five basic tastes and astringency. Nonetheless, initial sessions were conducted to further evaluate reference solutions in still bottled water (Sorgesana, Italy), corresponding to sour, sweet, bitter, salty, umami, and astringency sensations. These solutions (**Table S2**) were prepared in accordance with concentrations reported by the OIV[29]. Additionally, one session was devoted to a ranking test to assess the acidity intensity of tartaric acid solutions in still bottled water at 0, 0.25, 0.5, 0.75, and 1 g/L[29]. Ten specific sessions (conducted bi-

294 weekly for 2 hours each including discussion) were dedicated to training the panel on the concept
295 and complex sensations of kokumi. The initial sessions aimed to familiarise panellists with a
296 kokumi-active compound, namely GSH, at the concentrations reported in the literature [19, 31-33].
297 Specifically, GSH was added to still bottled water and white wine both alone and in combination
298 with other taste agents (**Table S3**). Additional sessions aimed to further familiarise panellists with
299 the kokumi concept and to recognise and describe the kokumi sensations in white wine using GSH
300 at three concentrations reported in wine (low: 10 mg/L, medium: 20 mg/L, high: 30 mg/L) [34]. For
301 further exercises in recognition and quantification, and to develop a consensus vocabulary for
302 kokumi sensations, Leu-Ala, a dipeptide reported to exhibit kokumi properties[19], was employed.
303 During this training phase, GSH (30 mg/L) and Leu-Ala at its threshold (708 mg/L= 3.1 mM) were
304 added to water, model wine (12% v/v EtOH, 6.3 g/L titratable acidity, with pH adjusted to 3.0 using
305 NaOH 1.0 M), and a sparkling base wine adjusted to two pH levels (3.0 with H_2SO_4 1.0 M and 3.8
306 with NaOH 1.0 M). The consensus vocabulary was developed based on descriptors and conceptual
307 references associated with the kokumi concept, and included kokumi and its flavour characteristics:
308 smoothness, harmony, mouthfulness, drying, and long-lasting flavour[6, 35].

309 A focus group was deemed necessary to further familiarise participants with the consensual
310 vocabulary, using the dipeptide under investigation, Gly-Val, at 9 different concentrations (ranging
311 from 3.75 to 180 mg/L) plus the control without Gly-Val in white wine (pH 3.2).

312 Finally, further training sessions were devoted to familiarisation with sensory test procedures and
313 scales, including triangle tests (TT), 3-alternative forced choice trials (3-AFC) and descriptive
314 analysis with relative to reference scaling (DA with RR scaling)[36], utilising a 15-cm line scale with
315 3 anchor points, low (=1.5), medium (=7.5= reference= white wine), and high (=13.5) intensities[37].

316 **2.3.5 Sensory analysis sessions.** TT[30]: trained panellists carried out triangle tests aimed at
317 exploring whether Gly-Val is orally discriminable in different wine matrices (model wine, white
318 wine, and sparkling base wine) detailed later. Six peptide solutions with an initial concentration of

120 mg/L of Gly-Val were prepared for each matrix. The samples were gradually diluted 1:1 (v/v) from 120 mg/L to 7.5 mg/L. An additional concentration of 90 mg/L was prepared.

3-AFC[38]: 10 selected subjects performed 3-AFC trials aimed at investigating the oral detection threshold (DT) of Gly-Val at 7.5, 15, 30, 60, and 90 mg/L, namely the point at which it could be detected that the matrix (model or real white wine) contained something other than itself, correctly identifying the odd sample within the triad for two consecutive replicates as described by Haryono et al[39].

DA with RR scaling[36]: 10 selected subjects tested the potential kokumi sensory effect of Gly-Val in white wine using DA with RR scaling. The in-mouth modulating effects of 15, 30 and 60 mg/L of added Gly-Val in white wine were evaluated. The fourth sample was white wine without added Gly-Val (0 mg/L). The panel was given a reference for comparison with the 4 samples during the evaluation and they were instructed to taste the reference before evaluation of each sample. The reference was the control wine without the addition of Gly-Val. During this phase, participants were asked to evaluate the following sensations: sweet, salty, sour and bitter tastes; flavour descriptors associated with the concept of kokumi and developed as a consensus vocabulary during training (smoothness, harmony, mouthfulness, drying, and long-lasting flavour); in general kokumi as a flavour-enhancing sensation. The samples were evaluated in 2 repetitions. The intensity of the attributes was assessed using a 15-cm scale on which panellists were previously trained. Panellists were informed that the mean anchor point (7.5) corresponded to the reference (white wine not spiked with Gly-Val).

2.3.6 Sensory statistical analysis. The significance ($\alpha \geq 0.05, 0.01, 0.001$) of the triangle test was determined according to ISO 4120:2021(E). Analysis of variance (ANOVA) and Tukey's HSD multiple comparison tests at 5% significance level were performed on data from DA with RR scaling using XLSTAT (2020.5) software (Addinsoft 2020). ANOVA was run on the differences with Gly-Val concentration as a fixed effect and the panellist as a random effect.

344

345 **2.4 Fermentation trials**

346 **2.4.1 Yeast strains.** Ten active dry yeasts for oenology, belonging to the *Saccharomyces* spp.
347 genus, and two type strains of non-*Saccharomyces* yeast having oenological interest
348 (*Hanseniaspora uvarum* and *Torulaspora delbrueckii)* were employed in this work. A detailed list
349 is provided in the Supporting information. Active dry yeasts were rehydrated prior to use according
350 to OIV standards, while the inoculum in the fermenting media (grape must, barley must, apple juice
351 or YM medium) was adjusted to reach a nominal concentration of 6 log units/mL. Type strains were
352 cultured in YM medium (Yeast Extract 3.0 g/L, Malt Extract 3.0 g/L, Peptone 5.0 g/L, Dextrose
353 10.0 g/L, both Oxoid, Basingstoke, UK).

354 **2.4.2 Experimental fermentation.** Four different media were tested in our experiments for
355 alcoholic fermentation: grape must cv. Chardonnay provided by the Edmund Mach Foundation
356 Winery (San Michele all'Adige, Italy) and used without any correction of sugars or nitrogen
357 content, barley must (Export Pilsner, Black Rock Brewing, Dunedin, New Zealand) prepared as
358 suggested by the producer, apple juice (Santàl, Parmalat s.p.a., Parma, Italy) and YM medium.
359 Fermentation was performed in glass bottles having a volume of 1 L at 20 ±1 °C and lasted 10 days.
360 During fermentation, the bottles were closed with a cap having a non-return valve to release the
361 CO_2 produced, avoiding excessive oxidation or microbial contamination of different batches.
362 Fermentation was followed daily with measurement of sugar content using an optical refractometer.
363 After sugar consumption the samples were stored at 3 ±1 °C to favour clarification for 48 hours in
364 closed bottles, then further analysed.

365 **2.4.3. Principal component analysis.** All zero values for the kokumi peptides were replaced by a
366 random number between 0 and the minimum measured value for the corresponding compounds.
367 PCA was performed on imputed, log-transformed and scaled data using the "FactoMineR" package
368 and visualisation of the generated model using the "factoextra" R package .

3. RESULTS

3.1 LC-MS method validation

Validation of the analytical protocol was conducted in duplicate using exactly the same methods for red and white wine. Since the performance was found to be substantially identical, without any significant deviations, it was considered sufficient to present in full only the dataset concerning white wines in the manuscript and supplementary information.

3.1.1 Linearity and Limit of Quantitation (LOQ). Calibration standards were evaluated at 16 concentration levels, prepared by diluting stock solution in methanol:water 1:1. A linear polynomial model was employed with the 1/X weighting factor (**Table S1**). A correlation coefficient (r2) greater than 0.990 was indicative of good linearity in a specific concentration range. The experimental LOQ value (**Table S1**) was calculated for each analyte by determining the lowest calibration point with a signal-to-noise (S/N) of 10. The LOD, corresponding to S/N=3, can be derived from the LOQ values. Wine peptide metabolites are present in different concentration ranges, from ppb to hundreds of ppm, but there is not much difference between red and white wine. Therefore, the calibration curves were specifically designed to cover the expected concentration in wines, after preliminary injections to see if the mass spectrometer could have signal saturation problems. Many points on the line were injected at different concentrations in order to cover a wide range of linearity. Generally speaking, the linearity range for all compounds covered four orders of magnitude, in the range between 0.002 and 5-10 mg/L, except for some compounds that went up to 25-30 mg/L before falling out of the desired linearity range (**Table S1**). A twenty-fold dilution of white and red wine enabled quantification of low abundant metabolites down to a few ppb, and at the same time prevented saturation of the detector by more abundant ones.

3.1.2 Matrix effect. The matrix effect was evaluated using matrix match calibration. For red and white wine, calibration curves at 16 concentration levels were prepared by diluting stock solution directly in red or white wine diluted with methanol/water 1:1. The percentage of the matrix effect was calculated as follows: (MMC slope MMS/SC slope) × 100. Acceptable ranges were 80−120%. The results obtained show that ME was in an

acceptable range (80−120%) for all metabolites in red and white wine**,** except for L-glutamic acid (79.4%) and L-histidine (79.9%).

3.1.3 Recovery. This parameter was evaluated using three different concentrations: low, medium, and high. The low and high concentrations were set as 4-fold lower and higher than the medium value found in the two wines (red and white) used for validation. Analytical recovery was assessed using the post standard addition method. For pre-processing (PRE-PR), the wine was filtered. For post-processing standard addition (POST-SP), spiking was conducted after the wine had been filtered. Recovery was expressed as:

% recovery = (PRE-PR concentration)/(POST-SP concentration) × 100.

Seven replicates were analysed for each concentration level. Analytical recovery in white and red wine was generally greater than 80%, with some exceptions, such as L- glutamine in red wine, with spikes at low concentration (122%), medium concentration (124%) and high concentration (133%). Other compounds that were slightly under 80% in red wine were Ala–Leu in the low spike concentration (70%) and Gly–Glu in the medium level concentration (71%). In white wine we obtained values similar to red wine. The exceptions in high spike concentrations were for L- glutamic acid (78%), L-isoleucine (76%) and L-leucine (77%). L-Val-Gly and Leu-Leu-Leu were slightly under 80% in all concentrations: high (68%) – (77%) medium (73%) – (78%) and low (78%) – (73%). Leu–Ale was 77% in the high spike concentration.

3.1.4 Intra and interday precision and accuracy. Intraday repeatability and accuracy were assessed by analysing samples (n= 7) spiked at medium concentration. Seven of the 95 metabolites we searched for (1 dipeptide; HPro-Pro, and 6 oligopeptides; N-Acetyl-Asp-Glu, Gln-Val-Gly, Arg-Gly-Asp-Ser, Glu-Cys-Me - Gln, Gln-Val-Gly-Met, and Gln-Val-Gly-Met -Glu,) were not considered since they were not present in the wines we used (values below LOD).

Interday repeatability and accuracy were assessed by analysing the same samples at days 3 and 5 (**Figure S1**). Precision is expressed as the coefficient of the variation percentage (CV%); for acceptance, CV% is required to be lower than 15%. Accuracy is expressed as a ratio (detected metabolite concentration/spiked concentration) × 100. The interday CV% result was below 15% for all metabolites detected in red and white wine, with the

exception of GSSG, due to the known instability of glutathione, and the tripeptide Asp-Asp-Val, present at trace level and having CV% 32.9%.

3.1.5 Metabolite stability. Metabolite stability was assessed by analysing samples (n= 5) spiked at medium concentration. These were tested at time 0 and after 7 days. The vials were left at 5 degrees in the autosampler before being reinjected. Stability was calculated as (average concentration at time X/average concentration at time 0) × 100. Overall variation was expressed as CV%, calculated considering all time points. After 7 days at 5 °C in the autosampler, the majority of metabolites in red wine and in white wine showed a stability of over 90%. The exceptions in white wine were γ-Glu-Cys and Ala-Pro (87%), and Asp-Asn-Val (88%). From these results we can say that the stability of the molecules analysed after 7 days can be considered to be excellent. The best results in terms of stability were seen in red wine.

3.1.6 Analytical performance during method validation. It was decided to use Trp-d5, a deuterated internal standard, to keep the sample dilution and filtration process monitored and under control. During the analysis sequence for the method, a mixed sample of white and red wine (QC) was injected every 10 injections. CV% for Trp-d5 in QC injected at fixed intervals was below 10%, indicating good analytical performance throughout all acquisition sequences.

3.1.7 LC-MS protocol application to commercial wines. The method was tested on sparkling wine and a total of 94 metabolites were detected and quantified, when present (**Table 2**). Of these, 76 compounds were found to be detectable, while the other analytes were below the limit of detection in Ferrari Perlè Trentodoc vintage Blanc de Blanc. The compounds quantified spanned over four orders of magnitude in their concentration, from an average of 293 mg/L for proline, down to 0.03 mg/L for the tripeptide Phe-Ser-Phe.

Table 2. List, in decreasing order of concentration, of the average concentration (avg) of amino acids and oligopeptides quantified in five vintages of a premium sparkling wine. The standard deviation (s.d.) is given for the analysis of three samples for each vintage. Data in mg/L.

	2017		2018		2019		2020		2021	
Name	avg.	s.d.	avg.	s.d.	avg.	s.d.	avg.	s.d.	avg.	s.d.
L-proline	292.02	4.44	280.80	1.89	302.46	1.67	303.24	2.34	286.84	5.36

L-pyroglutamic acid	60.23	1.05	59.25	0.81	70.91	0.96	69.22	0.87	49.52	1.28
L-glutamine	46.12	1.69	43.42	1.54	53.45	1.60	55.72	1.65	42.86	1.49
L-Lysine	47.87	1.87	41.59	1.29	53.88	2.76	52.81	1.37	45.19	1.99
L-glutamic acid	26.88	1.67	25.52	0.14	32.40	1.53	32.19	0.64	26.23	0.22
L-Leucine	26.10	0.48	23.47	0.49	29.58	0.81	29.88	0.45	20.87	1.06
L-Alanine	23.70	0.45	22.35	0.25	27.80	0.29	27.86	0.51	22.90	0.46
L-Asparagine	21.73	0.16	20.89	0.43	24.83	1.23	25.28	0.71	20.36	0.57
L-Phenylalanine	18.97	0.59	17.61	0.36	22.26	0.54	22.37	0.38	17.27	0.72
L-Arginine	25.34	1.11	11.01	0.45	24.34	0.08	24.15	0.59	10.26	0.85
L-Aspartic acid	19.60	0.66	16.81	0.19	19.39	0.25	20.07	0.39	13.75	0.27
L-Threonine	15.14	0.33	14.10	0.20	14.78	0.29	15.12	0.28	9.83	0.01
Asp-Leu	9.77	0.22	8.81	0.49	12.64	0.05	12.34	0.36	22.02	0.41
Glycine	13.27	0.10	10.70	0.10	13.41	0.70	12.55	0.33	10.18	0.54
L-serine	9.01	0.16	7.82	0.14	9.43	0.16	9.39	0.19	6.57	0.47
L-isoleucine	7.36	0.11	6.44	0.15	8.05	0.21	8.04	0.12	5.81	0.19
trans-4-hydroxy L-prolin	6.79	0.18	7.11	0.02	6.73	0.21	6.94	0.05	6.89	0.16
L-ornithine	10.72	0.26	5.48	0.25	5.81	0.28	5.56	0.15	5.26	0.07
L-Tyrosine	8.16	0.16	7.00	0.02	5.28	0.03	5.41	0.10	4.11	0.06
Asp-Val	5.62	0.19	5.19	0.11	6.29	0.17	6.29	0.15	5.65	0.17
L-citrulline	5.85	0.34	3.88	0.20	6.41	0.58	5.27	1.11	5.82	0.12
L-Norvaline	5.59	0.22	4.86	0.20	5.91	0.07	6.01	0.10	4.47	0.18
L-methionine	4.66	0.05	4.22	0.04	6.13	0.09	5.99	0.09	4.18	0.23
L-Valine	4.65	0.22	4.23	0.11	5.13	0.05	5.23	0.16	3.86	0.10
Ala-Pro	5.70	0.40	3.39	0.04	2.03	0.17	2.05	0.04	1.48	0.26
Ala-Lys	3.17	0.03	2.60	0.49	3.37	0.16	3.16	0.29	1.98	0.25
L -Histidine	4.29	0.07	2.89	0.03	2.62	0.04	2.68	0.03	0.66	0.09
Ala-Asp	2.61	0.08	2.09	0.02	1.66	0.03	1.66	0.03	0.90	0.06
Leu-Ala	1.66	0.11	1.28	0.06	1.39	0.04	1.38	0.11	0.77	0.09
Ala-Leu	1.54	0.05	1.14	0.04	1.20	0.04	1.13	0.04	0.78	0.03
L glutathione	0.19	0.01	0.22	0.01	0.83	0.02	0.86	0.02	2.17	0.08
Leu-Ser-Phe	0.80	0.02	0.76	0.01	0.95	0.01	0.95	0.00	0.74	0.02
Leu-Leu	0.93	0.02	0.85	0.02	0.83	0.01	0.83	0.01	0.55	0.02
Tyr-Ala	0.75	0.05	0.67	0.02	0.95	0.02	0.88	0.05	0.55	0.05
Leu-Pro	0.80	0.01	0.61	0.02	0.87	0.02	0.85	0.01	0.53	0.01
Leu-Leu-Leu	0.63	0.01	0.60	0.01	0.72	0.01	0.72	0.00	0.57	0.01
Gly-His	0.82	0.02	0.63	0.01	0.64	0.07	0.66	0.03	0.46	0.05
Leu-Gly	0.97	0.05	0.67	0.03	0.52	0.06	0.52	0.04	0.46	0.01
Asp-Asp-Val	0.64	0.13	0.61	0.04	0.66	0.10	0.69	0.08	0.44	0.11
Asp-Gly-Leu	0.60	0.06	0.55	0.04	0.59	0.03	0.59	0.06	0.49	0.02
Gly-Hval	0.50	0.05	0.47	0.03	0.59	0.05	0.65	0.06	0.59	0.04
Val - Gly	0.76	0.04	0.58	0.03	0.45	0.01	0.45	0.01	0.38	0.02
Pro-Thr	0.70	0.02	0.55	0.03	0.52	0.02	0.49	0.03	0.34	0.01
Tyr-Phe	0.42	0.01	0.41	0.01	0.62	0.01	0.63	0.01	0.45	0.02
Phe-Thr	0.63	0.02	0.53	0.02	0.50	0.01	0.51	0.02	0.31	0.01
Phe-Ala	0.52	0.03	0.47	0.03	0.58	0.01	0.59	0.02	0.31	0.02
Glu-Glu	0.32	0.02	0.38	0.01	0.65	0.02	0.64	0.06	0.42	0.01
Ala-Tyr	0.54	0.01	0.51	0.03	0.47	0.01	0.48	0.04	0.27	0.01

Ala-Phe	0.57	0.01	0.48	0.02	0.44	0.02	0.46	0.02	0.25	0.02
Asp-Tyr	0.50	0.02	0.46	0.02	0.42	0.02	0.44	0.03	0.33	0.02
Gly-Pro-Glu	0.44	0.02	0.38	0.01	0.42	0.02	0.42	0.01	0.28	0.02
L-cysteine	0.00	0.00	0.00	0.00	0.66	0.04	0.60	0.03	0.58	0.06
Gly-Asp	0.38	0.02	0.36	0.03	0.34	0.04	0.31	0.03	0.17	0.04
Asp-Ile	0.24	0.01	0.20	0.03	0.32	0.02	0.39	0.11	0.27	0.03
Asp-Phe	0.31	0.01	0.24	0.01	0.20	0.01	0.21	0.02	0.18	0.01
Pro-Leu	0.27	0.01	0.23	0.01	0.26	0.01	0.24	0.02	0.14	0.01
y-Glu-Val-Gly	0.31	0.03	0.28	0.02	0.19	0.01	0.20	0.01	0.11	0.01
L-tryptophan	0.07	0.06	0.04	0.07	0.20	0.01	0.20	0.01	0.56	0.02
Cyclo Met-Pro	0.39	0.01	0.25	0.01	0.17	0.00	0.17	0.01	0.09	0.00
Thr - Phe	0.22	0.01	0.20	0.01	0.24	0.01	0.23	0.02	0.17	0.01
Tyr-Phe	0.15	0.01	0.15	0.01	0.23	0.01	0.24	0.01	0.17	0.01
trans-4-hydroxy L-isoleucine	0.17	0.03	0.17	0.02	0.19	0.04	0.20	0.03	0.13	0.01
Asp-Gly-Ile	0.18	0.02	0.16	0.02	0.15	0.01	0.15	0.01	0.12	0.01
Gly-Glu	0.15	0.02	0.14	0.01	0.16	0.01	0.17	0.01	0.12	0.01
Thr-Pro	0.07	0.01	0.07	0.01	0.16	0.01	0.17	0.01	0.13	0.01
Gly-Val-Gly	0.12	0.00	0.10	0.01	0.09	0.01	0.11	0.01	0.08	0.01
Gly-Gly	0.10	0.01	0.09	0.01	0.12	0.01	0.13	0.02	0.07	0.00
Val-Gly-Gly	0.13	0.02	0.10	0.01	0.11	0.01	0.09	0.01	0.07	0.01
Pro-Met	0.09	0.00	0.09	0.01	0.09	0.01	0.10	0.01	0.07	0.00
Cyclo Gly-Pro	0.14	0.01	0.10	0.00	0.07	0.00	0.08	0.02	0.05	0.01
Asp-Gly	0.07	0.05	0.03	0.05	0.10	0.03	0.11	0.00	0.09	0.08
Phe-Thr-Phe	0.05	0.00	0.05	0.00	0.08	0.00	0.08	0.00	0.07	0.00
Met-Pro	0.06	0.01	0.04	0.00	0.07	0.01	0.08	0.01	0.05	0.01
Ile-Pro-Ile	0.06	0.00	0.06	0.00	0.07	0.01	0.07	0.01	0.04	0.00
Phe-Ser-Phe	0.02	0.00	0.02	0.00	0.03	0.00	0.03	0.00	0.05	0.01
Gly - Val	traces	n.d.	traces	n.d.	traces	n.d.	traces	n.d.	traces	n.d.
Asp-Met	<LOD	-	<LOD	-	<LOD	-	<LOD	-	<LOD	-
HVal-Gly	<LOD	-	<LOD	-	<LOD	-	<LOD	-	<LOD	-
HPro-Pro	<LOD	-	<LOD	-	<LOD	-	<LOD	-	<LOD	-
Asp-Asn-Val	<LOD	-	<LOD	-	<LOD	-	<LOD	-	<LOD	-
Asp-Gly-Val	<LOD	-	<LOD	-	<LOD	-	<LOD	-	<LOD	-
Cyclo Pro-Thr	<LOD	-	<LOD	-	<LOD	-	<LOD	-	<LOD	-
y-Glu-Cys1	<LOD	-	<LOD	-	<LOD	-	<LOD	-	<LOD	-
Arg-Gly-Asp-Ser	<LOD	-	<LOD	-	<LOD	-	<LOD	-	<LOD	-
Gln - Val - Gly	<LOD	-	<LOD	-	<LOD	-	<LOD	-	<LOD	-
Glu-Val-Gly	<LOD	-	<LOD	-	<LOD	-	<LOD	-	<LOD	-
Gln - Val - Gly - Met - Glu	<LOD	-	<LOD	-	<LOD	-	<LOD	-	<LOD	-
Glu - Cys - Met - Gln	<LOD	-	<LOD	-	<LOD	-	<LOD	-	<LOD	-
Gln - Val - Gly - Met	<LOD	-	<LOD	-	<LOD	-	<LOD	-	<LOD	-

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As many as seven dipeptides were found at a concentration between 13 and 1 mg/L, in decreasing order: Asp-Leu, Asp-Val, Ala-Pro, Ala-Lys, Ala-Asp, Leu-Ala, and Ala-Leu. A further 35 di- or tripeptides were found to be always present, at variable concentrations from 1 mg/L to 0.1 mg/L. In decreasing order, these were: γ-Glu-Cys-Gly (GSH), Leu-Ser-Phe, Leu-Leu, Tyr-Ala, Leu-Pro, Leu-Leu-Leu, Gly-His, Leu-Gly, Asp-Asp-Val, Asp-Gly-Leu, Gly-Hval, Val–Gly, Pro-Thr, Tyr-Phe, Phe-Thr, Phe-Ala, γ-Glu-Glu, Ala-Tyr, Ala-Phe, Asp-Tyr, Gly-Pro-Glu, Gly-Asp, Asp-Ile, Asp-Phe, Pro-Leu, γ-Glu-Val-Gly, Cyclo Met-Pro, Thr–Phe, Tyr-Phe, Asp-Gly-Ile, Gly-Glu, Thr-Pro, Gly-Val-Gly, Gly-Gly, and Val-Gly-Gly. Seven others were present at a concentration below 0.1 mg/L, while Gly-Val was confirmed but not quantified in this analysis. Indeed, the first application went smoothly for all the analytes, with the sole exception of the dipeptide Gly-Val. We initially had some difficulties with the quantification of Gly-Val because we noticed that at transition 175/129 an unknown compound was eluting in higher concentrations and at a close retention time. This led to problems of reliability and integration of the peaks. After tests carried out with the multiple addition method and MS^3 checks we were able to understand that the best compromise for quantification of this compound is transition 175/72 with a retention time of 2.74.

Our first observation therefore confirmed (**Table 2**) the presence of a mixture of 50 oligopeptides in the wine chosen for the experiment. The wine we selected for this part of the study was produced – at least for all the vintages selected - with a very rigorous and highly standardised methodology, to ensure that the whole process from the vineyards (always the same) to the bottle, including every phase of winemaking (juice extraction, stabilisation, first and second fermentation, etc), was carried under strict technical control, and supervised by the same "chef du cave", in order to deliver the desired oenological style. The three bottles sampled at the cellar from each vintage can be considered as technical replicates, while the five vintages are definitely biological replicates. It was somewhat surprising to note that the whole pattern of metabolites, both amino acids and oligopeptides, was closely similar in all the samples considered in this study (**Table 2**). The first conclusion is therefore that the profile of amino acids and oligopeptides in a vintage classic sparkling wine can be highly replicable when produced under closely controlled and standardised

conditions. The next step aimed to evaluate if any of the compounds detected could be candidates for kokumi properties.

The second survey conducted targeted a selection of 34 commercial Trentodoc sparkling wines from 29 producers, with the aim of including representative samples of the 67 Trentodoc wine cellars. They were aged on the lees for between 36 and 130 months, belonging to vintages from 2011 to 2019, including both Chardonnay and Pinot Noir or their blends, and a single sample of Pinot Blanc. In this part of the study we focused our attention on the presence of glutamic acid and 11 putative kokumi oligopeptides (see section 3.2 for specific discussion).

L-Glutamic acid in Trentodoc wines ranged between 19.55 and 83.72 mg/L **(Table 3**) with an average concentration of 46.89 mg/L. This content is in agreement with the typical values for wines, as summarised in the literature [10]. Half of the sparkling wines (17 out of 34) had a glutamic acid concentration exceeding the reported taste threshold of 48 mg/L in white wines [11]. This suggests that the glutamic acid content in Trentodoc sparkling wines is in a range sufficient to impart an umami taste, also in agreement with conclusions in the literature, based on the sensory trial for red wines [10].

Table 3. L-glutamic acid and 11 putative kokumi peptides quantified in 34 samples of vintage Trentodoc sparkling wine. Data in mg/L

Cultivar (%)	aging on lees (months)	Year	Style	Color	L-glutamic acid	L glutathione	Asp-Val	Ala-Pro	Ala-Lys	Asp-Leu	Leu-Ala	Ala-Asp	Glu-Glu	Asp-Gly-Leu	Asp-Ile	Gly-Val	Asp-Gly-Ile	sum of oligop.
100% Chardonnay	130	2011	Extra Brut	white	45.23	0.11	4.72	2.99	3.61	2.44	2.66	3.81	0.21	0.40	0.29	0.17	0.18	**17.80**
100% Chardonnay	96	2015	Brut	white	43.96	0.34	4.64	1.86	4.19	2.40	2.36	2.71	0.17	0.38	0.20	0.12	0.14	**16.80**
100% Chardonnay	70	2016	Extra Brut	white	55.44	0.34	7.69	4.99	3.37	2.00	2.87	2.62	0.18	0.31	0.20	0.15	0.15	**22.23**
100% Chardonnay	60	2018	Brut	white	71.53	3.64	6.97	4.85	3.51	3.37	2.48	2.07	0.19	0.42	0.22	0.08	0.15	**25.89**
100% Chardonnay	60	2016	Extra Brut	white	58.59	0.19	10.08	5.39	1.44	3.17	2.53	2.23	0.17	0.16	0.17	0.14	0.05	**23.48**
100% Chardonnay	60	2016	Extra Brut	white	52.25	0.18	5.23	0.46	2.97	3.59	1.87	2.82	0.26	0.28	0.13	0.11	0.10	**15.17**
100% Chardonnay	55	2018	Brut	white	42.55	0.18	6.49	2.91	2.23	3.32	2.11	2.84	0.21	0.29	0.24	0.10	0.05	**18.13**
100% Chardonnay	53	2018	Brut	white	26.99	0.16	9.49	5.37	1.81	3.80	1.39	1.27	0.09	0.18	0.04	0.05	0.12	**22.48**
100% Chardonnay	50	2018	Extra Brut	white	34.96	0.30	6.14	3.09	2.58	2.40	3.07	2.59	0.40	0.23	0.18	0.11	0.09	**18.59**
100% Chardonnay	48	2017	Extra Brut	white	71.76	0.27	11.27	5.69	3.72	2.62	5.05	3.76	0.24	0.47	0.40	0.26	0.14	**30.14**
100% Chardonnay	48	2018	Zero Dosage	white	29.77	0.47	4.59	1.75	1.97	1.90	1.30	1.58	0.25	0.20	0.15	0.06	0.12	**12.76**
100% Chardonnay	48	2017	Zero Dosage	white	63.48	0.27	9.12	3.24	3.48	3.28	4.69	3.98	0.44	0.42	0.33	0.24	0.19	**25.70**
100% Chardonnay	41	2019	Brut	white	19.55	0.37	0.92	0.10	2.40	2.54	1.78	1.91	0.33	0.26	0.20	0.05	0.11	**9.05**
100% Chardonnay	36	2019	Zero Dosage	white	37.22	0.46	6.81	2.12	3.87	3.07	3.47	2.09	0.40	0.30	0.23	0.09	0.11	**20.93**
100% Chardonnay	36	2019	Extra Brut	white	49.73	0.08	14.04	5.69	5.45	3.63	2.93	3.16	0.66	0.34	0.19	0.10	0.17	**33.29**
100% Chardonnay	36	2019	Zero Dosage	white	83.72	0.26	7.68	3.92	1.18	3.30	2.20	2.02	0.19	0.17	0.16	0.15	0.09	**19.28**
100% Pinot Blanc	42	2019	Extra Brut	white	33.20	0.37	5.43	2.13	2.30	2.29	1.68	1.39	0.24	0.23	0.07	0.08	0.19	**15.02**
100% Pinot Noir	90	2015	Zero Dosage	white	49.28	0.09	7.67	4.33	2.05	2.92	2.75	2.62	0.19	0.27	0.14	0.12	0.08	**20.61**
100% Pinot Noir	90	2015	Brut	rosè	61.24	0.13	6.82	2.54	2.32	2.66	2.99	3.28	0.20	0.28	0.14	0.12	0.12	**18.34**
100% Pinot Noir	60	2017	Brut	rosè	23.47	0.11	5.41	2.61	2.01	4.15	1.91	2.21	0.31	0.20	0.15	0.11	0.13	**17.09**
100% Pinot Noir	60	2017	Extra Brut	white	37.04	0.07	4.66	2.25	1.62	2.25	1.45	1.97	0.18	0.16	0.12	0.07	0.07	**12.88**
100% Pinot Noir	50	2018	Zero Dosage	white	51.67	1.89	0.03	0.44	2.50	2.77	2.59	3.14	0.45	0.21	0.27	0.12	0.09	**11.35**
100% Pinot Noir	50	2018	Extra Brut	white	34.87	0.15	10.28	8.47	2.86	1.99	1.37	1.74	0.32	0.23	0.13	0.05	0.09	**25.96**
100% Pinot Noir	42	2019	Zero Dosage	rosè	42.06	1.21	4.47	0.15	2.26	1.84	2.95	2.90	0.34	0.32	0.20	0.07	0.18	**13.97**
100% Pinot Noir	36	2020	Brut	rosè	54.83	1.92	5.15	2.83	3.76	2.09	2.40	2.29	0.74	0.35	0.33	0.17	0.10	**19.84**
100% Pinot Noir	36	2019	Brut	rosè	31.01	1.19	3.79	1.78	2.06	2.43	1.28	1.10	0.25	0.20	0.21	0.05	0.11	**13.33**
100% Pinot Noir	36	2019	Extra Brut	white	27.27	0.10	5.53	2.46	1.78	2.19	1.37	1.53	0.21	0.26	0.11	0.07	0.11	**14.21**
50% Pinot Noir, 25% Chardonnay, 25% Pinot Blanc	70	2017	Extra Brut	white	50.08	0.32	6.08	3.19	2.09	3.78	2.88	2.76	0.45	0.25	0.22	0.11	0.09	**19.46**
52% Chardonnay 48% Pinot Noir	40	2019	Zero Dosage	white	47.63	0.21	11.45	6.02	2.12	2.37	2.16	2.66	0.11	0.27	0.18	0.17	0.11	**25.18**
60% Chardonnay, 40% Pinot Noir	48	2018	Zero Dosage	white	41.33	0.13	6.42	3.69	5.01	3.14	2.55	3.15	0.23	0.47	0.13	0.11	0.22	**22.10**
68% Chardonnay, 32% Pinot Noir	43	2019	Brut	white	66.08	0.97	8.06	3.48	5.04	2.36	5.77	4.12	0.95	0.73	0.49	0.15	0.19	**28.19**
80% Chardonnay, 20% Pinot Noir	60	2018	Zero Dosage	white	51.19	4.16	5.42	2.75	2.09	3.24	1.57	2.13	0.31	0.29	0.15	0.10	0.06	**20.15**
80% Chardonnay, 20% Pinot Noir	60	2017	Extra Brut	white	55.85	0.06	9.04	8.48	2.57	2.28	3.87	3.88	0.45	0.44	0.24	0.16	0.20	**27.77**
85% Chardonnay, 15% Pinot Noir	80	2016	Brut	white	49.57	0.21	5.73	3.30	1.50	2.16	2.30	2.23	0.17	0.23	0.22	0.11	0.02	**15.94**

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Looking at the 11 putative kokumi peptides, these were ubiquitous, being above the LOQ in all the wines analysed. The most abundant were the five dipeptides Asp-Val, Ala-Pro, Ala-Lys, Asp-Leu and Leu-Ala, but a further six peptides were always present (**Table 3**). The total concentration of putative kokumi peptides in these sparkling wines varied by a factor of 3.7, i.e. between 9.05 and 33.29 mg/L, with average values of 19.80 mg/L. No statistically significant difference was observed between 100% Chardonnay and Pinot Noir products. In contrast with the first dataset, where only a single brand was investigated, leading to a highly coherent pattern and concentration, in this second dataset, which included several producers and types, we observed relatively wide variability, requiring future study to investigate possible sources of variability (cultivars, yeasts, fining, etc.).

3.2. Molecular modelling

Table 4. Docking score unit amounts* (PLPScore scoring function) of considered sequences

Sequence	**Docking Score (PLPscore)**
Ala-Val[a]	**81**
Leu-Ala[b]	**89**
Ala-Asp	**75**
Ala-Leu	**86**
Ala-Lys	**97**
Ala-Phe	**92**
Ala-Pro	**79**

Ala-Tyr	**97**
Asp-Gly	**80**
Asp-Ile	**95**
Asp-Leu	**96**
Asp-Met	**94**
Asp-Phe	**98**
Asp-Tyr	**99**
CycloGly-Pro	**70**
CycloMet-Pro	**90**
CycloPro-Thr	**91**
Glu-Glu	**101**
Gly-Asp	**72**
Gly-Glu	**78**
Gly-Gly	**72**
Gly-His	**83**
Gly-Hval	**79**
Gly-Val	**79**

Hpro-Pro	**75**
Hval-Gly	**84**
Leu-Gly	**87**
Leu-Leu	**99**
Leu-Pro	**89**
Met-Pro	**86**
Phe-Ala	**99**
Phe-Thr	**99**
Pro-Leu	**82**
Pro-Met	**83**
Thr-Phe	**101**
Thr-Pro	**86**
Tyr-Ala	**100**
Tyr-Phe	**106**
Val-Gly	**80**
Asp-Asn-Val	**105**
Asp-Asp-Val	**113**

Asp-Gly-Ile	**111**
Asp-Gly-Leu	**107**
Asp-Gly-Val	**106**
Gln-Val-Gly	**104**
Gly-Pro-Glu	**100**
Gly-Val-Gly	**93**
Ile-Pro-Ile	**111**
Leu-Ser-Phe	**119**
Phe-Ser-Phe	**124**
Phe-Thr-Phe	**118**
Val-Gly-Gly	**94**

3.2.1 Model validation. As shown in **Table 4,** assuming proper interaction with the VFT domain to be the event triggering receptor activation, all the peptides recorded positive and relatively high scores, pointing to a possible ability to satisfy the physicochemical requirements of the pocket (the higher the score, the better the ligand-pocket match; see section 2.2 for further details). However, analysis of the docking poses revealed different behaviour in the sequences in terms of occupying the binding site. Specifically, the di-peptide Leu-Ala, taken as a reference positive control (see section 2.2.1 for further details), displayed non-covalent interaction with residues Ala168, Glu297 and Gly273 (**Figure 1B**). Interestingly, one of these residues (Ala168) was also involved in the binding architecture of γ-Glu-Val-Gly, which is the most potent CaSR-activator γ-Glutamyl tripeptide

511 described *in vitro* to date [23] (**Figure 1A**). Conversely, the inactive sequence Ala-Val only showed an
512 ability to interact with Ala168, while no other polar interactions were observed, pointing to possibly
513 less stable interaction with the pocket (**Figure 1B**). As stated above, stabilisation of the closed
514 conformation of the VFT domain is a fundamental mechanism for CaSR activation and kokumi taste
515 perception. Therefore, the distances between the α-carbons of Arg66-Ser303, Pro274-Phe42, and
516 Tyr246-Val44 induced by the different ligands were measured to describe VFT opening/closure
517 (**Figure 1C**), in line with a previous study[18]. The output collected revealed that the Leu-Ala kokumi-
518 active sequence kept the three regions (C1, C2, and C3) closed during the whole simulation, while all
519 the interatomic distances considered increased constantly during the simulation, when in complex
520 with the negative control Ala-Val (**Figure 1D**). These results highlight the success of the 3D
521 molecular model used here to discriminate kokumi-active sequences from inactive ones. Indeed, these
522 results are in line with those reported for strong activators and kokumi-active molecules such as γ-
523 Glu-Val-Gly and L-Trp, and conversely for inactive sequences[18].

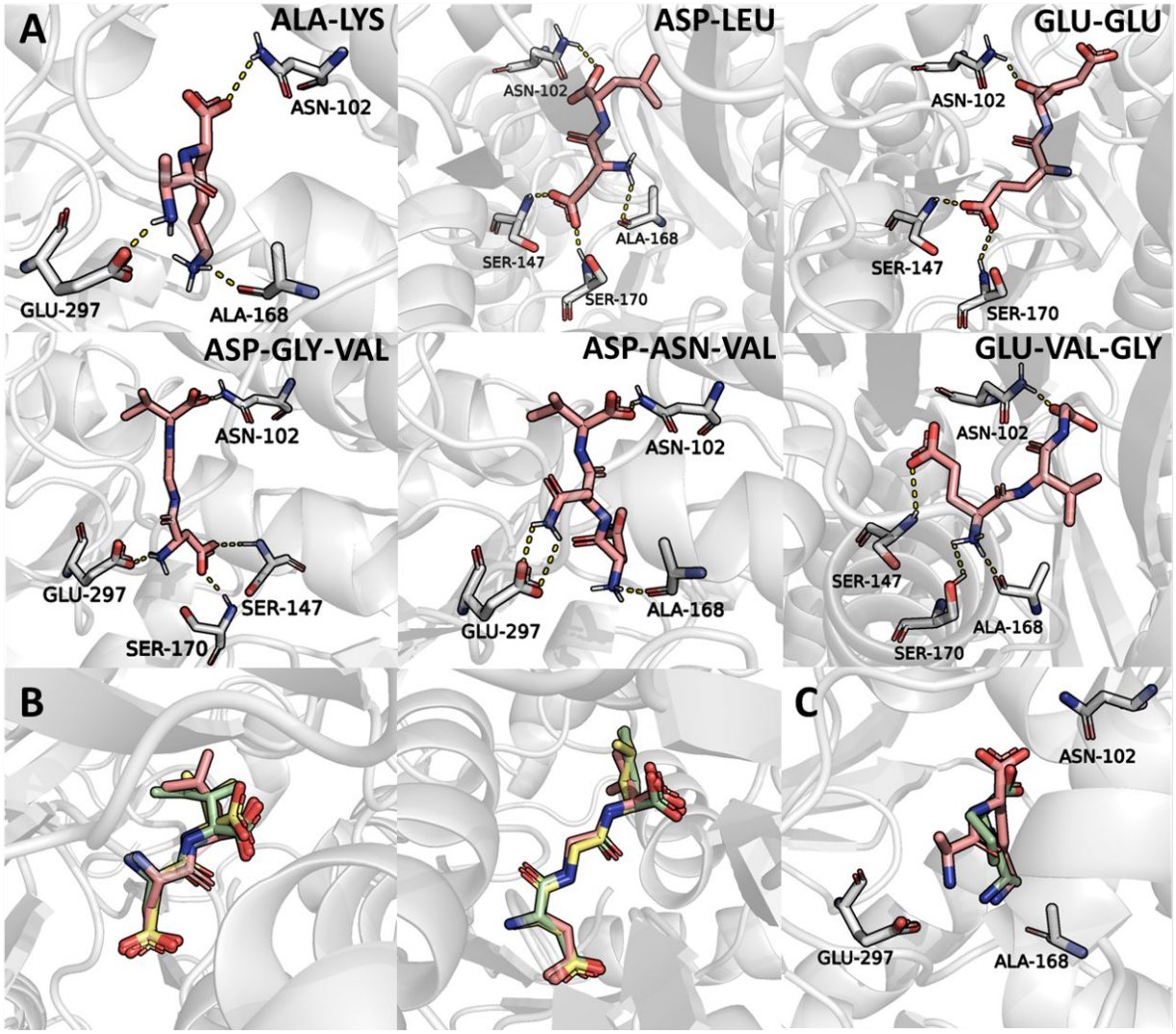


Figure 2. Docking poses of the peptides under analysis. The protein is represented in cartoon, ligands and amino acids involved in polar interactions are represented in sticks, and polar interactions are represented as yellow dashed lines. **A.** The pose of Ala-Lys, Asp-Leu, Glu-Glu, Asp-Gly-Val, Asp-Asn-Val and Glu-Val-Gly are shown. **B.** On the left, superimposition of Asp-Leu (salmon), Asp-Ile (green) and Asp-Val (yellow), on the right superimposition of Asp-Gly-Val (salmon), Asp-Gly-Ile (green) and Asp-Gly-Leu (yellow). **C.** Binding pose of Ala-Pro in comparison to Ala-Lys.

3.2.2 Analysis of wine sequences. Once fit-for-purpose validation was achieved, the sequences identified in wine samples were calculated via docking simulation. It is worth noting that one subset showed binding architecture similar to that of the two reference kokumi-active sequences Leu-Ala

535 and γ-Glu-Val-Gly (**Figures 1A-B** and **Figures 2A-B**). This suggests they might interact with VFT
536 as activators, and therefore they were carried forward to MD simulation to study the stability of the
537 complexes over time. As shown in **Figures 2A** and **2B**, this subset included five dipeptides (namely,
538 Ala-Lys, Asp-Leu, Asp-Ile, Asp-Val, and Glu-Glu) and five tripeptides (Asp-Gly-Val, Asp-Gly-Leu,
539 Asp-Gly-Ile, Asp-Asn-Val, and Glu-Val-Gly), never accounted before for their kokumi taste, to the
540 best of our knowledge. Considering the strong similarity between Asp-Leu, Asp-Ile and Asp-Val, and
541 Asp-Gly-Val, Asp-Gly-Leu and Asp-Gly-Ile (**Figure 2B**), one representative of each subset (Asp-
542 Leu and Asp-Gly-Val) was further calculated with MD simulations, assuming comparable behaviour
543 for the respective congeners, along with Ala-Lys, Glu-Glu, Asp-Asn-Val and Glu-Val-Gly.
544 Furthermore, the peptide Ala-Pro, which showed a pose similar to Ala-Lys though the Pro moiety,
545 could not engage the protein, likewise Lys in Ala-Lys (**Figure 2C**) was also included in the study
546 because of its relatively high level in wine (**Table 5)** and its partial similarity with Ala-Lys in
547 occupying the binding site. Since Gly-Val was selected as the referencing sequence for sensory tests
548 but its quantification in a first LC-MS analysis was impossible due to interference by a coeluting
549 compound, this di-peptide was included in the calculations to verify its capability to act as kokumi
550 active peptide. The best scoring docking poses of Ala-Lys, Asp-Leu, Glu-Glu, Ala-Pro, Gly-Val,
551 Asp-Gly-Val, Asp-Asn-Val and Glu-Val-Gly were used as AD input for MD simulations.

552

553 **Table 5**. Average amounts of the sequences of interest detected in Ferrari Perlè (5 vintages).

Sequence	**Activity**	**2017**	**2018**	**2019**	**2020**	**2021**
Ala-Val[a]	- -	/	/	/	/	/
Leu-Ala[b]	+ +	1.66	1.28	1.39	1.38	0.77
Ala-Lys	+	3.17	2.6	3.37	3.16	1.98

Ala-Pro[c]	+	5.7	3.39	2.03	2.05	1.48
Asp-Leu	+ +	9.77	8.81	12.64	12.34	22.02
Asp-Ile[d]	+ +	0.24	0.2	0.32	0.39	0.27
Asp-Val[d]	+ +	5.62	5.19	6.29	6.29	5.65
Glu-Glu	+ +	0.32	0.38	0.65	0.64	0.42
Gly-Val	+ +	tr.	tr.	tr.	tr.	tr.
Asp-Asn-Val	+ +	<LOD	<LOD	<LOD	<LOD	<LOD
Asp-Gly-Val	+ +	<LOD	<LOD	<LOD	<LOD	<LOD
Asp-Gly-Leu[e]	+ +	0.6	0.55	0.59	0.59	0.49
Asp-Gly-Ile[e]	+ +	0.18	0.16	0.15	0.15	0.12
Glu-Val-Gly	+ +	<LOD	<LOD	<LOD	<LOD	<LOD

554

555 Legend: [a] not in the list, used as negative control; [b] in the list, used as positive control; [c] similarity
556 with Ala-Lys and abundance in wine; [d] similarity with Asp-Leu; [e] similarity with Asp-Gly-Val;
557 tr.=present in traces, n.d.=below LOD

558

559 The distances between the α-carbons of Arg66-Ser303, Pro274-Phe42, and Tyr246-Val44 of CaSR
560 when in complex with these oligopeptides were measured and compared to those recorded for Leu-
561 Ala and Ala-Val (**Figure S2**). The results pointed to the capability of all the considered sequences to
562 favourably interact with the pocket and keep the VFT domain tightly closed over time (**Figure S2**).

Of the analysed sequences, Ala-Lys was the only one responsible for partial instability of VFT domain closure at the end of the simulation (**Figure S2**). As discussed above, monitoring intra-molecular distances may provide semi-quantitative comparison of ligands in terms of activity [18]. On this basis, Ala-Lys could act as a weaker activator of the kokumi receptor compared to other sequences. Importantly, as reported in **Tables 2 and 3,** some of these oligopeptides (i.e. Ala-Pro, Asp-Leu and Asp-Val) are among those most abundant in Trentodoc wines in the different years considered and might have an important role in the kokumi flavour of these products.

3.3 Sensory analysis

This part of the experiment was a preliminary exploratory study on the sensory impact of Gly-Val. This compound is among putative ubiquitous kokumi peptides in wines, never previously reported in the literature as a kokumi substance, and its presence in wines is found in trace amounts, typically around 0.1-0.2 mg/L. A preliminary study was therefore conducted to explore and possibly reject the possibility that Gly-Val could act as a kokumi substance in white wine. Considering the scale of interaction with the CaSR protein, we could expect that if Gly-Val has kokumi properties, the other 10 peptides characterised by higher affinity with the CaSR protein binding sites, and present at a higher concentration in wine, would also have a good chance of being collectively sensorially bioactive. Gly-Val was arbitrarily considered as representative of the pool of investigated peptides and therefore tested in the concentration range detected.

Of course, more detailed studies are needed, requiring synthesis of adequate amounts of a mixture of the different kokumi substances representative of the pattern detected in wines, and possibly also considering performing sensory tests with different concentrations of glutamic acid, and this is a limitation of this preliminary sensory investigation. Three different matrices were used for sensory trials, as reported in **Table 6**.

588 **Table 6**. Results of the triangular tests performed on three wine matrices added with different
589 amounts of Gly-Val.

	Ethanol	Total Acidity	pH	n. subjects	Added Gly-Val (mg/L)						Odd sample correctly identified
Matrix	(% v/v)	(g/L)			7.5	15	30	60	90	120	(% at concentration in bold)
model wine	12	6.30	3.0	32	ns	ns	ns	ns	ns	**	57
white wine	10.5	6.10	3.2	26	ns	***	***	***	ns	nd	73
sparkling base wine	12	6.37	3.0	16	ns	ns	**	*	***	ns	75

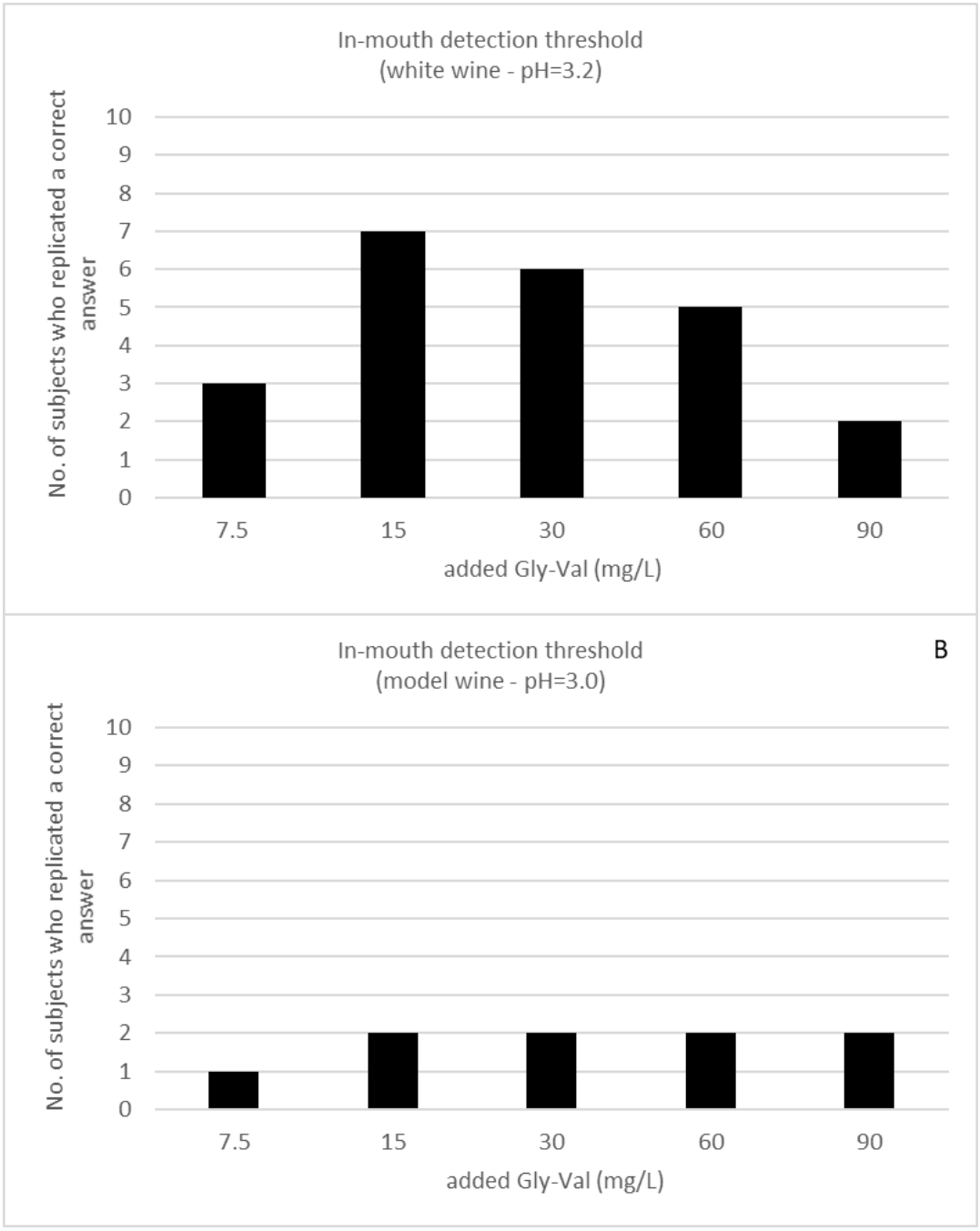
590

591 The results show that the significance of the triangle test varied according to the matrix and Gly-Val
592 concentration. The tested levels were arbitrarily chosen not as representative of the detected trace
593 amount of Gly-Val but as representative of the detected average level of the pool of investigated
594 peptides. In the model wine the odd sample was correctly identified only at the highest
595 concentration of 120 mg/L Gly-Val ($\alpha \geq 0.01$). In both real wines, the test was significant at lower
596 concentrations, in the range of 15-60 mg/L Gly-Val in the white wine ($\alpha \geq 0.001$) and 30-90 mg/L
597 Gly-Val in the sparkling base wine ($0.05 \geq \alpha \geq 0.001$). This suggests that the odd samples spiked with
598 Gly-Val were more discriminable in more complex matrices such as real wines compared to the
599 model wine. Moreover, the discriminability of the added dipeptide at different concentrations in the
600 white wine and sparkling base wine could be linked to their compositional differences. Smoother
601 conditions in terms of ethanol (10.5 % v/v), total acidity (6.10 g/L) and pH (3.2) in white wine

602 compared to the sparkling base wine (12 %v/V, 6.37 g/L, 3.0, respectively) seemed to facilitate
603 Gly-Val perception. The glutamic acid and kokumi peptide composition of the two wines used for
604 the trial are reported in **Table S4**. Interestingly, in both wines the concentration of L-glutamic acid
605 (41.28 and 52.41 mg/L) could follow the peri-threshold of MSG, whose threshold in wine has been
606 reported as 48 mg/L [11].

607 The results obtained in the 3-AFC test support this evidence. Indeed, after two testing sessions we
608 found that the DT for Gly-Val ranged from 15 to 60 mg/L (0.09 to 0.34 mM) (**Figure 3A**) in real
609 white wine, while in the model wine (**Figure 3B**) it was not possible to identify a DT range at the
610 investigated concentrations. For white wine, the maximum number of replicated correct answers (7
611 out of 10) was at 15 mg/L Gly-Val, followed by a decreasing trend at 30 and 60 mg/L Gly-Val (6 and
612 5 out of 10, respectively). This behaviour highlights nonlinear sensory activity at increasing peptide
613 concentrations.

614



615

616 **Figure 3**. Results of 3-AFC test representing the number of subjects correctly identifying the
617 sample spiked with Gly-Val at a given concentration (7.5, 15, 30, 60 and 90 mg/L) in two
618 consecutive test repetitions

619 Based on these results, the potential kokumi sensory effect of Gly-Val in white wine was tested using
620 DA with RR scaling. The in-mouth modulating effects of 15, 30 and 60 mg/L of added Gly-Val in
621 white wine were evaluated. The ANOVA test did not highlight any significant variation for the in-
622 mouth descriptors, however, some considerations can be made by looking at the trends shown in

Figure 4. The kokumi descriptor trend supports the awareness of subjects as regards kokumi perception, indeed they were repeatable compared to the reference, and the intensity trend follows the same behaviour as the in-mouth detection threshold at the same concentrations (**Figure 3A**). Gly-Val tended to modulate white wine taste by slightly raising sourness and bitterness from 30 mg/L, and mouthfeel sensations of smoothness and mouthfulness from 15 mg/L. The lack of significant differences could be at least partially linked to the limited sensitivity of DA with RR scaling compared to classic DA[36]. On the other hand, this method was supportive in terms of repeatability, probably because comparison with the reference was helpful in the evaluation of a complex and acquired sensation such as kokumi. These preliminary results open up the possibility of future in-depth sensory studies.

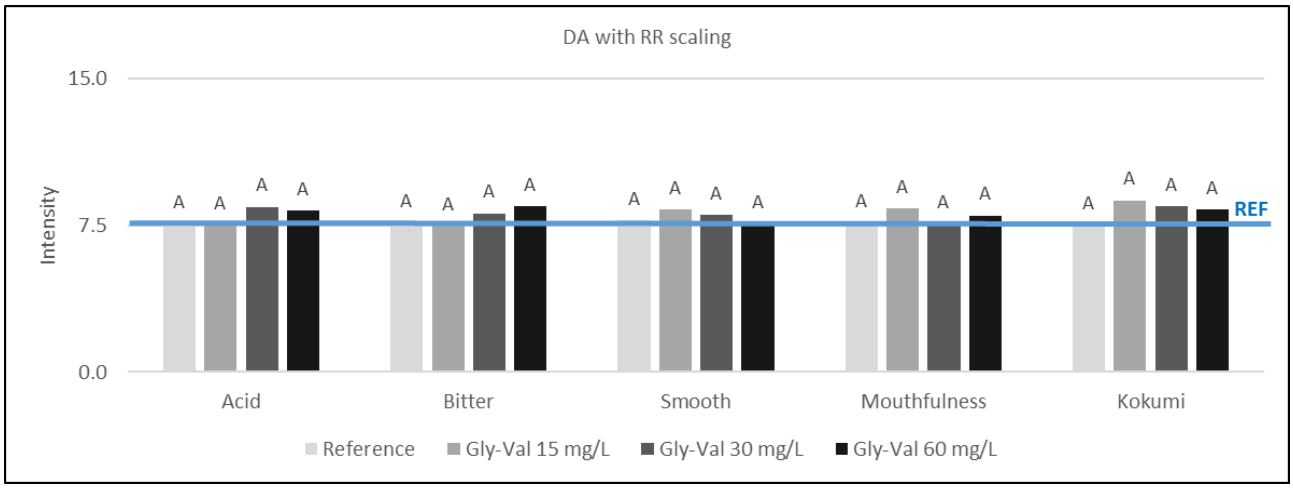


Figure 4. Mean intensity of in-mouth descriptors measured for each sample by DA with RR scaling. Letters refer to significant differences ($p \leq 0.05$) between samples within each descriptor. The horizontal light-blue line represents the reference (REF: added Gly-Val 0 mg/L) set at 7.5.

Overall, these preliminary results support the hypothesis that Gly-Val is a potential kokumi-active dipeptide. A kokumi-active compound is intended as a compound able to modulate in-mouth sensations due to other sensory active molecules, especially in complex matrices compared to simple ones [6]. In accordance with this, it was possible to significantly discriminate the presence of Gly-Val

642 and to estimate its detection threshold range in real white wines but not in model wine, suggesting
643 that it may only be active in the presence of other compounds present in real wine but missing in
644 model wine.

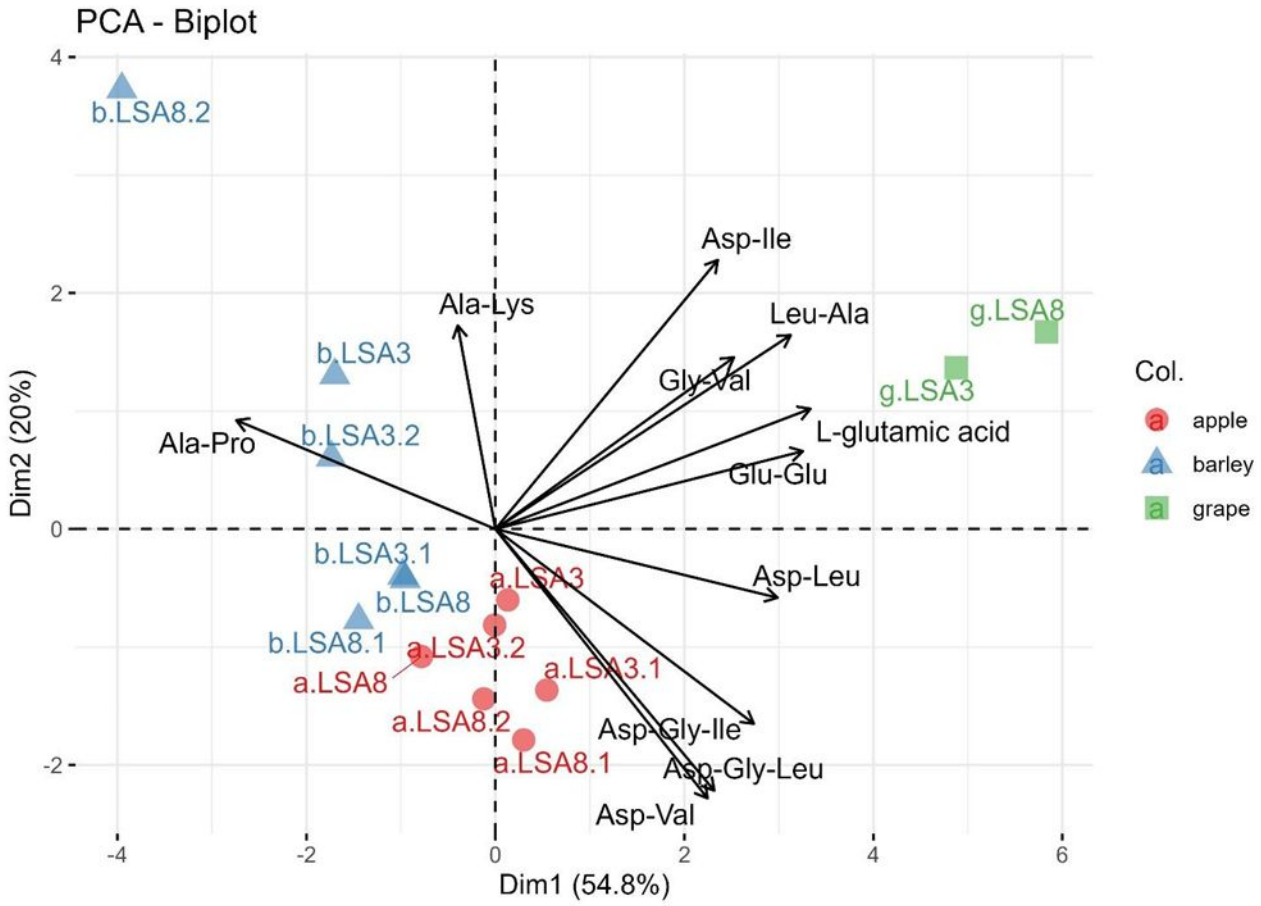
645 **4. Experimental fermentation**

646 The first fermentation test with different selected yeasts highlighted the formation of profiles and
647 concentrations that were decidedly different depending on the matrix investigated. The profile
648 resulting from fermentation of a defrosted Chardonnay must, therefore poor in proteins, was found to
649 produce small quantities of kokumi peptides, much lower than those found in samples of commercial
650 sparkling wines with an identical qualitative profile and with minute differences between different
651 yeasts in terms of the quantities of peptides produced (**Table S5**). This test will be repeated in the
652 future, starting with fresh must that better represents a real case scenario.

653 It was more interesting to observe the profile of fermented products obtained with YM medium.
654 Among the peculiarities of products obtained by fermenting the YM medium base, with two of the
655 three yeasts used (LSA3 and LSA10), it was possible to observe the formation of significant quantities
656 of Gly-Val (between 5 and 9 mg/L), together with similar concentrations of Leu-Ala, for fermentation
657 with LSA3 (**Table S6**). This high Gly-Val value, which is several dozen times higher than that found
658 in commercial wines, was checked several times to exclude interference, and analysis of the peak
659 confirmed its identity and purity.

660 Fermentation of three different matrices with a pair of selected dry yeasts highlighted that the pattern
661 of metabolites resulting in fermented beverages depended closely on the starting raw materials. The
662 differences are clearly visualised in the results of principal component analysis based on kokumi
663 peptides (**Figure 5**). Three tripeptides were found only in wines (Asp-Gly-Leu, Asp-Gly-Ile, and Glu-
664 Val-Gly), three dipeptides were significantly more abundant in wines (Leu-Ala, Glu-Glu, and Asp-
665 Leu), one dipeptide was present in significant concentrations both in beer and wine (Ala-Lys) while
666 only one dipeptide was dominant in beer samples (Ala-Pro) where it reached significant

667 concentrations, in the range of 10.74-12.55 mg/L. Only two dipeptides were also present in non-
668 negligible concentrations in fermented apple samples (Asp-Leu and Ala-Pro) while a further 4 were
669 present only in trace amounts. As principal component analysis performed on the kokumi peptide
670 profile (**Figure 5**) clearly shows, fermented beverages obtained from the three juices were well
671 separated for the first two principal components.



672

673 **Figure 5.** Principal component analysis based on the putative kokumi peptides, the scatterplot of the
674 cases and of the variables on the first two dimensions is showing how the fermentation of three
675 different matrices with a pair of selected dry yeasts (LSA3 and LSA8) has produced a different pattern
676 of the investigated peptides, depending closely on the starting raw materials.

677

678 The presence of kokumi peptides, albeit in low quantities, (**Table S7**) in the barley wort used for the
679 fermentation tests could be due to the native proteolytic activity of the barley seed, commonly
680 exploited in the malting and mashing phases. Furthermore, the fermented product has higher
681 concentrations of these compounds, confirming the key role of the yeast activity in the accumulation
682 of kokumi peptides. Another indirect confirmation of this hypothesis is found by observing that apple
683 juice, which does not undergo any "biological" degradation process (enzymatic or microbial), does
684 not contain kokumi compounds.

685 In conclusion, the results of the first pilot fermentation study agree with the hypothesis that
686 proteolysis or autolysis during food fermentation generates taste-active amino acids and peptides
687 (Zhao et al., 2016). The final profile and concentrations of kokumi peptides we analysed depended
688 strongly on the matrix, generating different patterns for wine, beer and cider. Consequently, in the
689 future it will be necessary to further investigate the role of technology, pre-fermentative fining, and
690 yeast nutrition in making proteins available as a substrate for the production of oligopeptides during
691 fermentation. There also seems to be a role for yeast, which certainly deserves future investigation in
692 the search for modulating the presence of kokumi compounds in fermented beverages. We believe
693 that this article represents a contribution to starting a line of study that will increase our understanding
694 and control of wine flavour.

695

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702 **Supporting information:** Additional experimental details, materials and methods (PDF)

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828 **Graphic for table of content**

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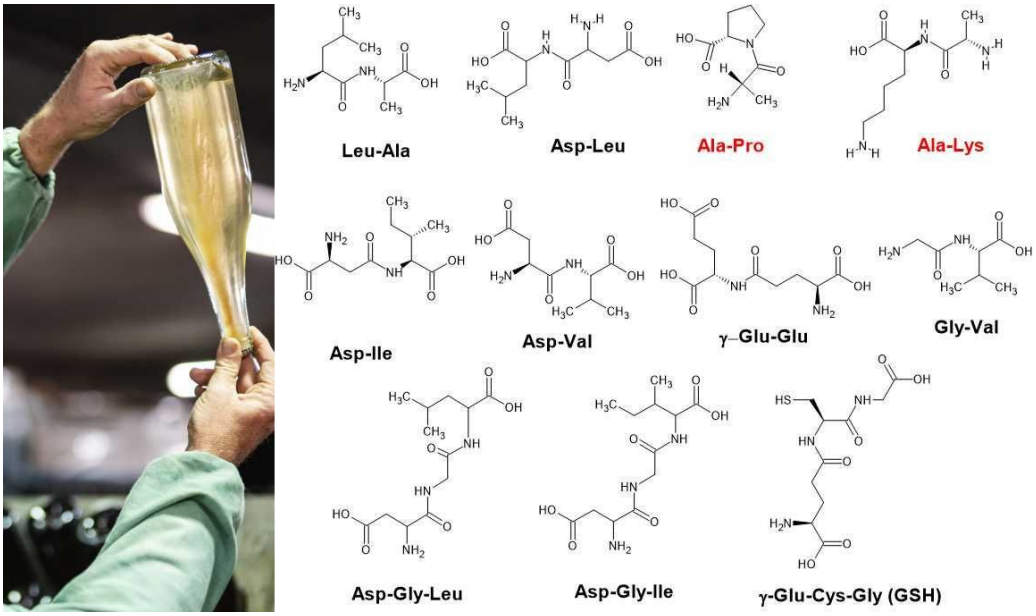

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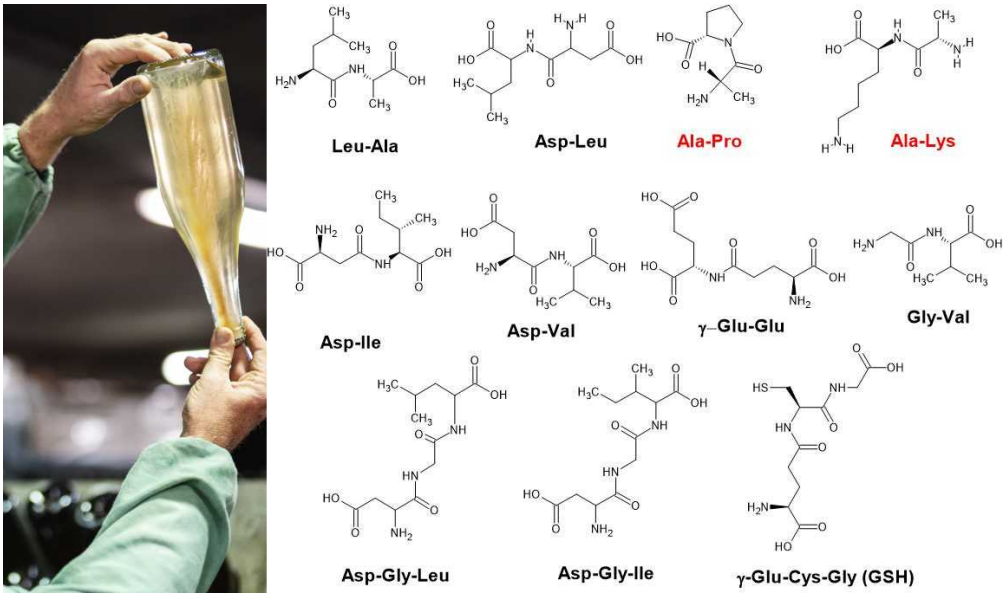

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